Trends in Analytical Chemistry 197 (2026) 118717

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Analytical approaches to uncover the hidden identity of non-extractable organic soil contaminants - a synopsis

Karolina M. Nowak [a,*], Jan Schwarzbauer [b], Andreas Schäffer [c,d,e,**]

[a] University of Koblenz, Environmental Geochemistry, Institute for Integrated Natural Sciences, Universitätsstraße 1, Koblenz, 56070, Germany
[b] RWTH Aachen University, Institute for Organic Biogeochemistry in Geo-Systems, Lochnerstrasse 4-20, 52056, Aachen, Germany
[c] RWTH Aachen University, Institute for Environmental Research, Worringerweg 1, 52074, Aachen, Germany
[d] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, 210093, Nanjing, China
[e] Key Laboratory of the Three Gorges Reservoir Region's Eco-Environment, Chongqing University, 400044, Chongqing, China

A B S T R A C T

Environmental fate assessment of organic contaminants in soil is essential for chemical risk assessment within the regulatory frameworks. While conventional biodegradability testing allows quantification of extractable residues and mineralisation, the analysis of non-extractable residues (NER) remains a significant analytical challenge. This review synthesizes current analytical strategies and workflows for the characterization and differentiation of NER in soil originating from organic contaminants.

Therefore, we describe current analytical strategies and workflows that enable robust NER characterization. The analytical framework distinguishes three NER types based on formation mechanisms and environmental relevance: type 1 (physically entrapped residues sequestered within soil aggregates with remobilization risk), type 2 (covalently bound residues with strong chemical bonds to a solid matrix and minimal remobilization potential), and type 3 (microbial biomass residues integrated into natural organic matter with no environmental concern).

Sequential extraction procedures using aqueous and organic solvents, combined with targeted chemical degradation methods and derivatisation like silylation, provide systematic approaches to distinguish NER types. Silylation enables the release and quantification of physically entrapped (type 1) NER, while remaining covalently bound (type 2) NER require chemo- or thermochemolytic cleavage for identification. Biogenic NER (type 3) are quantified via acidic hydrolysis and analysis of the released amino acids as microbial biomass proxies carrying the label from the degraded contaminant.

Isotopic labelling utilizing both radioactive (^{14}C, ^{3}H: tritium) and stable (^{13}C, ^{15}N, ^{2}H: deuterium) isotopes has greatly advanced mass balance tracking and molecular identification capabilities. While radioactive labelling provides unparalleled sensitivity for quantification, stable isotope methods coupled with GC-IRMS, LC-MS/MS, or NMR offer mechanistic and structural information. So far, NER research using isotope labelling was mainly based on ^{14}C-, ^{13}C- and ^{15}N, but recently the suitability of ^{2}H-labels for this purpose has been described. This innovative approach, which can ease distinction between the formations of xenobiotic NER (type 1 and 2) and biogenic NER (type 3), is presented here.

By integrating state-of-the-art extraction, degradation, and isotope-tracing techniques, researchers can now achieve robust discrimination of NER types. These analytical advancements are crucial for regulatory bodies to assess chemical safety, support persistence classification, and predict long-term risks associated with organic pollutants in soils. Suggestions for further research on the use of deuterium and tritium as isotope labels and the investigation of mass-dependent isotope effects on the degradation of the labelled test substance are also given.

1. Biodegradability testing of organic chemicals

The worldwide production of synthetic chemicals is rising and nowadays about 350,000 chemicals and mixtures of chemicals have been registered for commercial use [1]. Once released to the environment, organic chemicals can be transported to different compartments, volatilised, degraded biologically or abiotically, taken up by plants, and be immobilised in the soil or sediment matrix [2]. Easily biodegradable contaminants are eliminated from the environment whilst hardly biodegradable ones can accumulate in the environment for decades posing a hazard to living organisms [3].

The knowledge about the fate of organic chemicals in the environment is crucial for safeguarding the public and environmental health. Authorities across the world like the European Chemicals Agency

This article is part of a special issue entitled: Emerging contaminants in Soils published in Trends in Analytical Chemistry.

* Corresponding author.
** Corresponding author. RWTH Aachen University, Institute for Environmental Research, Worringerweg 1, 52074, Aachen, Germany.
E-mail addresses: knowak@uni-koblenz.de (K.M. Nowak), jan.schwarzbauer@emr.rwth-aachen.de (J. Schwarzbauer), andreas.schaeffer@bio5.rwth-aachen.de (A. Schäffer).

https://doi.org/10.1016/j.trac.2026.118717
Received 22 July 2025; Received in revised form 29 November 2025; Accepted 30 January 2026
Available online 4 February 2026
0165-9936/© 2026 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

(ECHA) thus implement numerous legislations (e.g., REACH [4]) requiring up-front laboratory testing of chemicals prior to their approval. Each manufacturer of any chemical to be produced at the amount of ≥10 tons/year has to test it accordingly for persistency (P), bioaccumulation potential (B) and toxicity (T) [5]. The persistence of a given chemical is assessed in biodegradability testing according to OECD Test Guidelines for Chemicals in a surface water, waste water, soil or water-sediment [6]. An isotope labelled test chemical is added to a reference system in order to quantify accurately the isotope distribution within the system (so-called *isotope mass balance* see Fig. 1) over the course of maximum 120-day incubation.

The isotope label in mineralisation products (e.g., CO_2, N_2, H_2O), extractable residues of untransformed chemical or its transformation products, and in the remaining solid matrix as non-extractable residues (NER, also called 'soil-bound' residues) make up the isotope mass balance [8]. While the mineralisation and extractable residues can be easily quantified and identified using analytical techniques, the NER assessment is often a 'black box' due to analytical challenges in their chemical identification [2,9].

Mineralisation and NER results provide a baseline for the P-assessment [4]. For instance, a high mineralisation value (>70%, within a certain time range, for instance within 7 days in the Zahn-Wellens test) indicates low 'P' of a given chemical. Contrastingly, low mineralisation (<5%) together with the NER formation above 70% indicates that a chemical is not readily biodegraded, but a significant portion is converted into NER. This can question the chemical safety, especially when the chemical identity of the NER is unknown. Therefore, a detailed chemical identity of the NER is essential for the P-assessment of organic contaminants in the regulatory biodegradability testing.

2. Non-extractable residues (NER): definition, types and related risks

The 'NER' term evolved during the last four decades and relies on the quantitation of a remaining isotope label in the solid matrix after the extraction of contaminant and its transformation products. For example, the IUPAC [10] defined the NER in 1984 as 'species originating from chemicals that remain un-extracted by methods that do not alter their chemical nature'. Fourteen years later, the NER term was refined to 'compounds in environmental matrices persisting as the parent substance or transformation products after non-destructive extraction [11]. At that time, the bioavailability of NER was commonly investigated. For that, soil containing NER was mixed with a fresh soil and the mineralisation of NER due to microbial degradation was quantitated. Another approach applied hydrolytic and pyrolytic methods to release residues, but "the problem of identifying soil NER remained as yet unsolved" [10].

According to a recent classification, NER 'are residues that remain in soil after exhaustive extraction of contaminants adsorbed onto the reactive surfaces of the solid matrix'. The NER can be formed either during physico-chemical interactions of the contaminants with the solid matrix or be a result of abiotic and microbial degradation and comprise xenobiotic NER (Xeno-NER) and biogenic NER (Bio-NER) [12]. The NER formation is generally influenced by physico-chemical properties of the contaminant, i.e., its molecular structure, reactivity and functional groups, and of the solid matrix such as clay and organic matter contents, pH, as well as microbiological activity [9]. All chemicals form NER in soils to various extents regardless of their chemical structure and functionalities [9,13]. Even hardly or non-degradable substances having no functional groups prone for binding to humic matter (-OH, –COOH, $–NH_2$) can build up NER due to the various interaction mechanisms (Fig. 2). Also high molecular weight polymers like microplastics can form non-extractable residues (on-going own research project) by interacting with soil aggregates along with organic matter, microbes, and soil mineral colloids, but methods quantifying microplastic-derived NER are still lacking [14].

Currently, the NER are grouped into three types based on formation mechanisms (Fig. 3) [9].

The first two are accordingly Xeno-NER type 1 and Xeno-NER type 2. Type 1 NER are contaminant or its transformation products that are 'locked up' in the tiny pores of soil aggregates (called here 'physically entrapped') [9]. Type 2 NER are contaminants chemically bound to reactive groups of the solid matrix via different strong covalent bonds, for example ester, amide, ether or carbon-carbon linkages (see Fig. 2); methods to determine various covalent binding modes of NER are

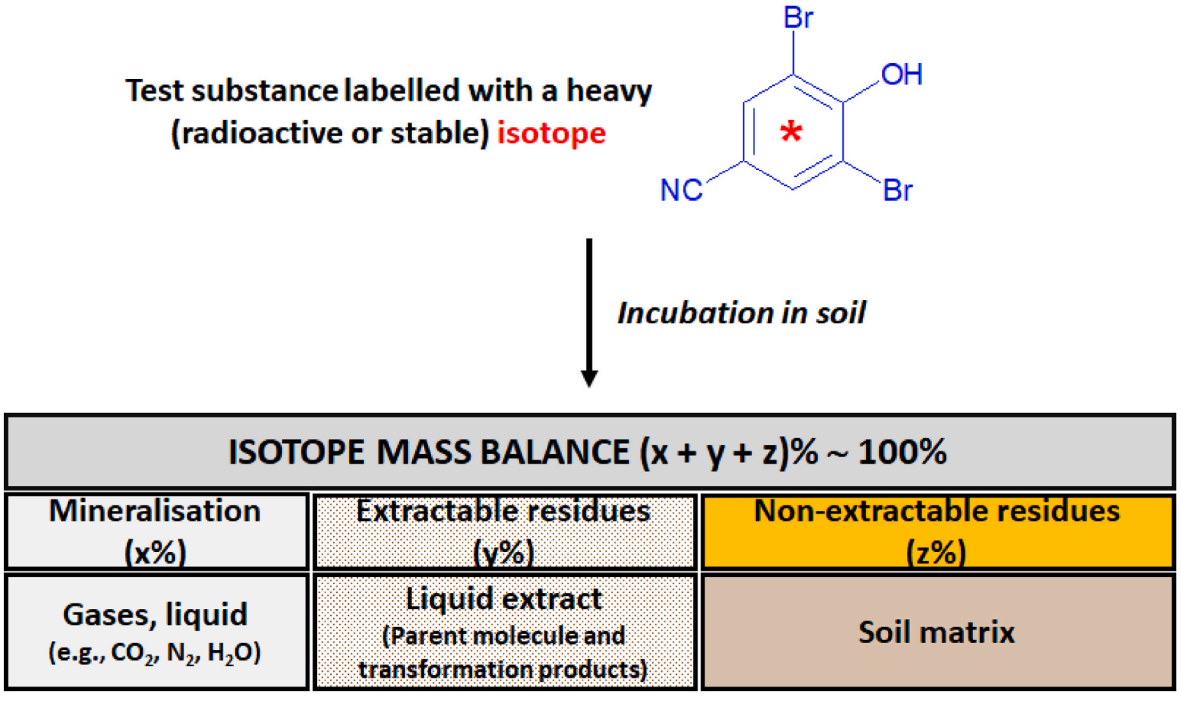


Fig. 1. Fate study of a test substance (here: the herbicide bromoxynil) in soil. The isotope mass balance comprises mineralisation (x%), extractable (y%) and non-extractable (z%) residue and should be around 100%. Percentages x, y, z depend on incubation time and may increase with time [7].

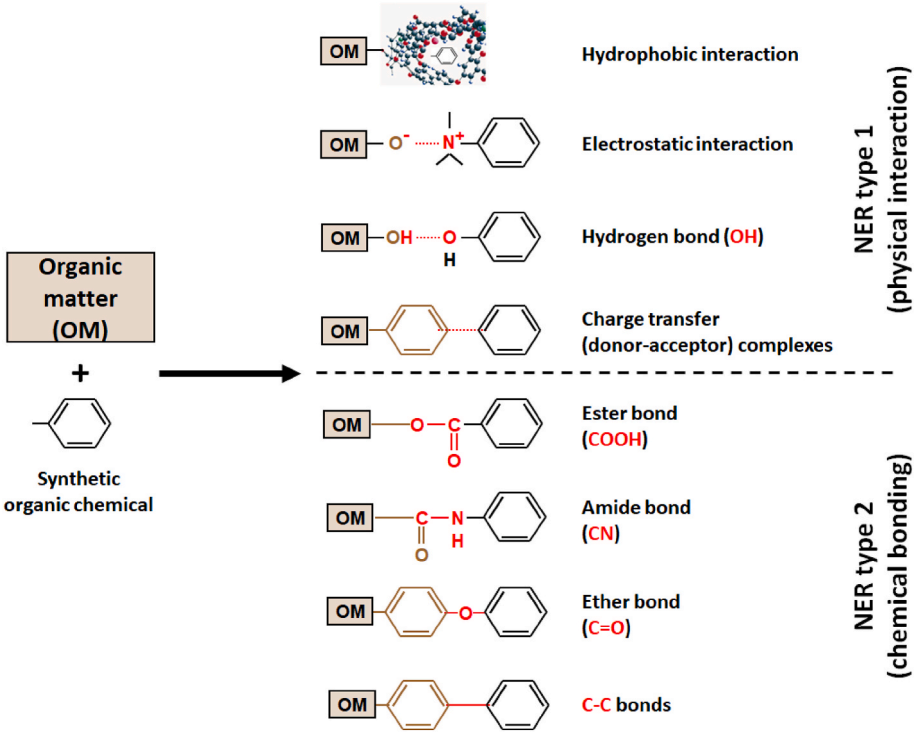


Fig. 2. Binding of a contaminant or its transformation products with the solid matrix. Here, we differentiate types of covalent bonds (Xeno-NER type 2) from physical interactions, i.e., adsorbed onto or entrapped into solids (Xeno-NER type 1). Methods for determination of covalent binding modes are described in chapter 6.

described in chapter 6[15,16]. Please note that absorbed chemicals onto solids as well as NER type 1 do not always form typical strong covalent bonds (Xeno-NER type 2), but weak bindings like hydrogen bonding, electrostatic and hydrophobic interactions or charge-transfer complexes [17] (type 1). These contaminants can be detached from the solids using specific extraction methods (for details see chapter 4).

Type 3 NER (Bio-NER) contain only biomolecules like amino acids or fatty acids, which are derived from the biomass of microbial degraders (Fig. 3 [3]). Microorganisms can exploit contaminant-elements like C, N or H for their biomass build-up during growth. After death, biomass residues (necromass) can be either recycled by other microorganisms or stabilised in the solid matrix forming biogenic NER. More details on Bio-NER characterisation are included in chapter 8. To sum this up, each NER type differs in environmental risk: type 1 poses remobilization concerns, type 2 is persistent, and type 3 is ecologically benign.

To differentiate type 1 and type 2 NER, silylation of the soils organic matter has been used [18] and is briefly described in chapter 5. Type 1 NER are of environmental concern because of their remobilization risk in the course of time, although the toxicity of such NER is considered lower than that of extractable residues as tested with the toxic compounds 2,4,6-trinitrotoluene (TNT), cypermethrin and carbendazim [19]. Type 2 NER have low to no remobilization potential due to the strong covalent bonds to the solid matrix (Fig. 3) [9]. Type 3 NER are environmentally of no concern as these comprise natural compounds that are integral part of organic matter produced naturally in soils or sediments [9]. Discriminating Xeno-NER from Bio-NER through isotope-labelling is necessary for the P-assessment of contaminants (see chapter 3) and the related remobilization risk, especially under changing climate conditions. A recent study demonstrated that even permafrost soils in the polar regions store large amounts of toxic contaminants like halogenated organic compounds, mostly in the form of NER type 1 with a remobilization risk [20]. The remobilization risk of toxic contaminants from such soils due to increasing temperatures is worrisome. Therefore, analytical approaches aiming at the distinction between the three NER types are crucial for preventive risk assessment of chemicals to be placed on the market, and these are described in detail in next sections.

3. Relevance of NER in persistence assessment

The inclusion of NER has a significant effect on the persistence assessment of chemicals. Degradation half-lives of chemicals depend on the assumptions whether (a) only the amounts of the extractable parent compound in the time course of a degradation experiment, or (b) the parent compound plus the total amount of NER as non-degraded parent is considered, if no further identification is performed, or (c) whether parent plus xenoNER (= the sum of types 1 and 2 NER or = total NER minus Bio-NER), or (d) the parent compound plus only type 1 NER is used for half-life calculations. Obviously, the first scenario (a) delivers shortest half-lives, which so far is common practice, and (b) the longest half-lives, which overestimates persistence because total NER remains undifferentiated and may contain no or only traces of the parent molecule. Since type 1 NER are prone to be released over time, calculations of the half-life based on the parent substance plus this fraction (case (d)) will yield most realistic results (Fig. 4).

Due to the impact of NER in calculating half-lives, the European Chemicals Agency (ECHA) updated the guidance on persistence (P), bioaccumulation (B) and toxicity (T) and asks for the inclusion of NER, emphasizing the importance of advanced NER characterization for regulatory decision-making. However, the experimental studies to differentiate NER types are laborious. Another way to approach corrected half-lives is by modelling the formation of Bio-NER using the Microbial Turnover to Biomass (MTB) model [21] and subtracting this amount from the total amount of NER resulting in the amount of Xeno-NER, although using this way residues are not identified [22].

4. Extraction techniques to isolate extractable and non-extractable residues

Prior to the analysis of NER, a two-step extraction procedure must be

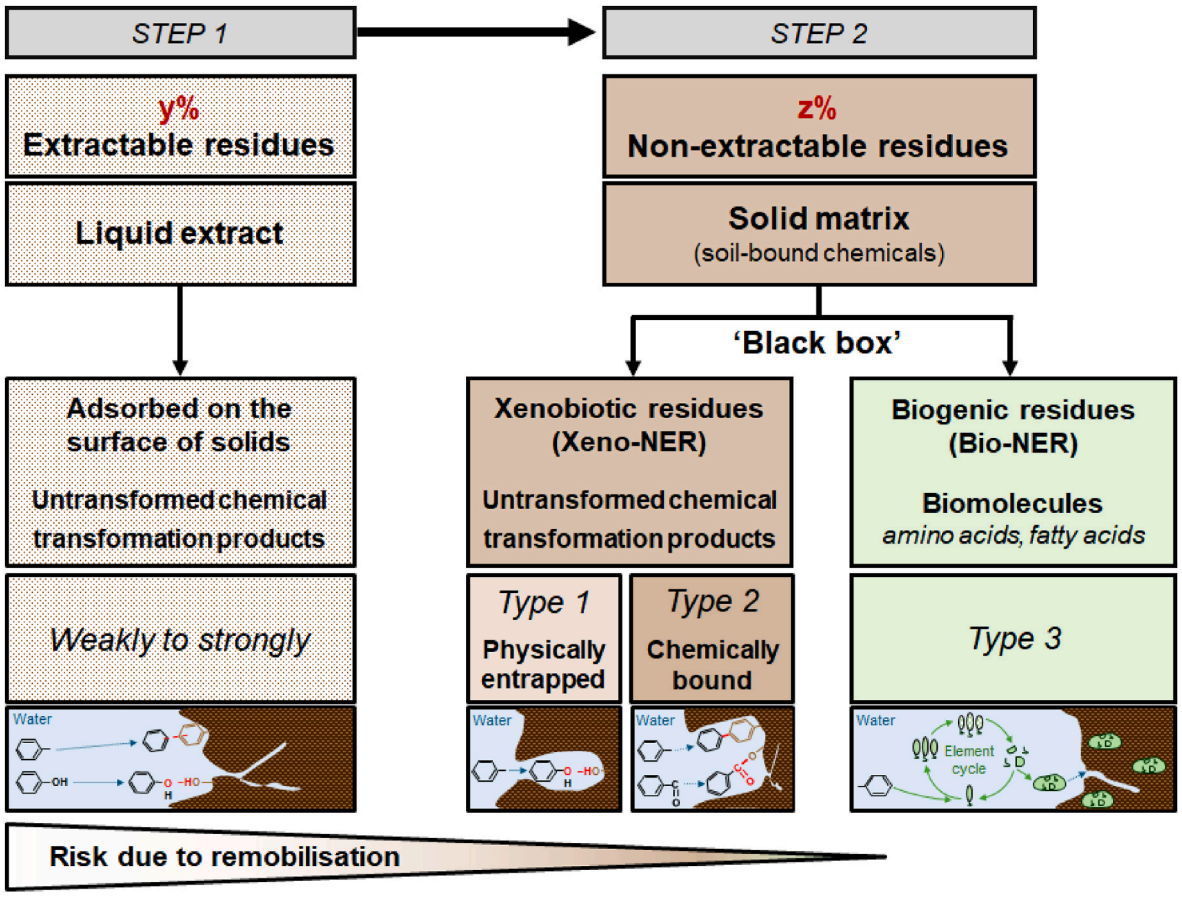


Fig. 3. Extractable and non-extractable residues of a contaminant in solid matrices. The isotope label in non-extractable residues (NER, step 2) is quantified after the solvent-extraction of the contaminant residues that are weakly to strongly adsorbed onto the reactive surfaces of the solid matrix (step 1). The NER can contain xenobiotic residues (Xeno-NER) of either type 1 (physically entrapped) or type 2 (chemically bound), as well as biogenic residues (Bio-NER). y% and z% refer to Fig. 1 in which x% is the mineralisation amount.

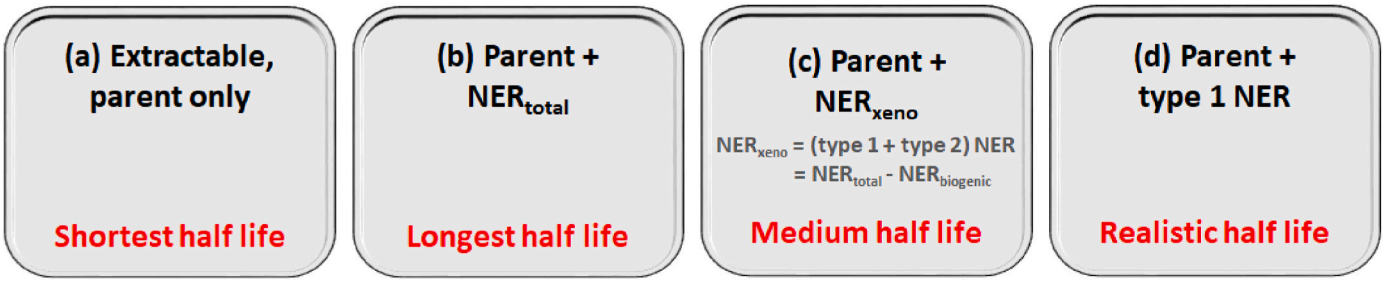


Fig. 4. Persistence assessment scenarios of chemicals in soil considering NER. Longest half life is obtained if NER has not been characterized assuming that it contains the parent compound (case (b)), the shortest half life, if only the extractable parent compound is considered (case (a)). Verification of the parent compound in type 1 NER in silylation or EDTA extracts would result in a realistic half-life (case (d)).

elaborated to isolate extractable resides from environmental matrices such as soil, sediment, and biological tissues (see Fig. 5) [9]. This extraction procedure also aims at the assessment of bioavailability and bioaccessibility of contaminants to living organisms [23]. The bioavailability refers to a freely available contaminant to an organism, while bioaccessibility encompasses both the bioavailable and the potentially bioavailable contaminant (Fig. 6). The bioaccessible contaminant can become bioavailable, for example; after the desorption processes or soil aggregate disruption, both triggered by environmental conditions [23].

Assessing bioavailability employs a combination of chemical and biological methods [23,24] (see Table 1). The chemical methods involve the three-step approach: assessment of 'freely' dissolved contaminants in ambient solutions (*step 1*), 'mild' (*step 2*) and 'harsh' (*step 3*) solvent extractions (Fig. 5). In the first step, the dissolved contaminants in the ambient solution or upon the addition of 0.01 M $CaCl_2$ - to mimic soil pore water chemistry - to a sample are directly quantified, and these are bioavailable [25].

The second step involves an extraction of contaminants at ambient-temperature using mixtures of water and organic solvents (e.g., water-acetonitrile mixture), sometimes enhanced with formic acid, acetic acid, or ammonia to improve their solubility depending on the chemicals functional groups. Prolonged physical agitation or ultrasonication further enhances extraction. The selection of solvent combinations depends on the physicochemical properties of the contaminant, e.g., solubility, pK_a, stability as well as on matrix properties (soil, sediment,

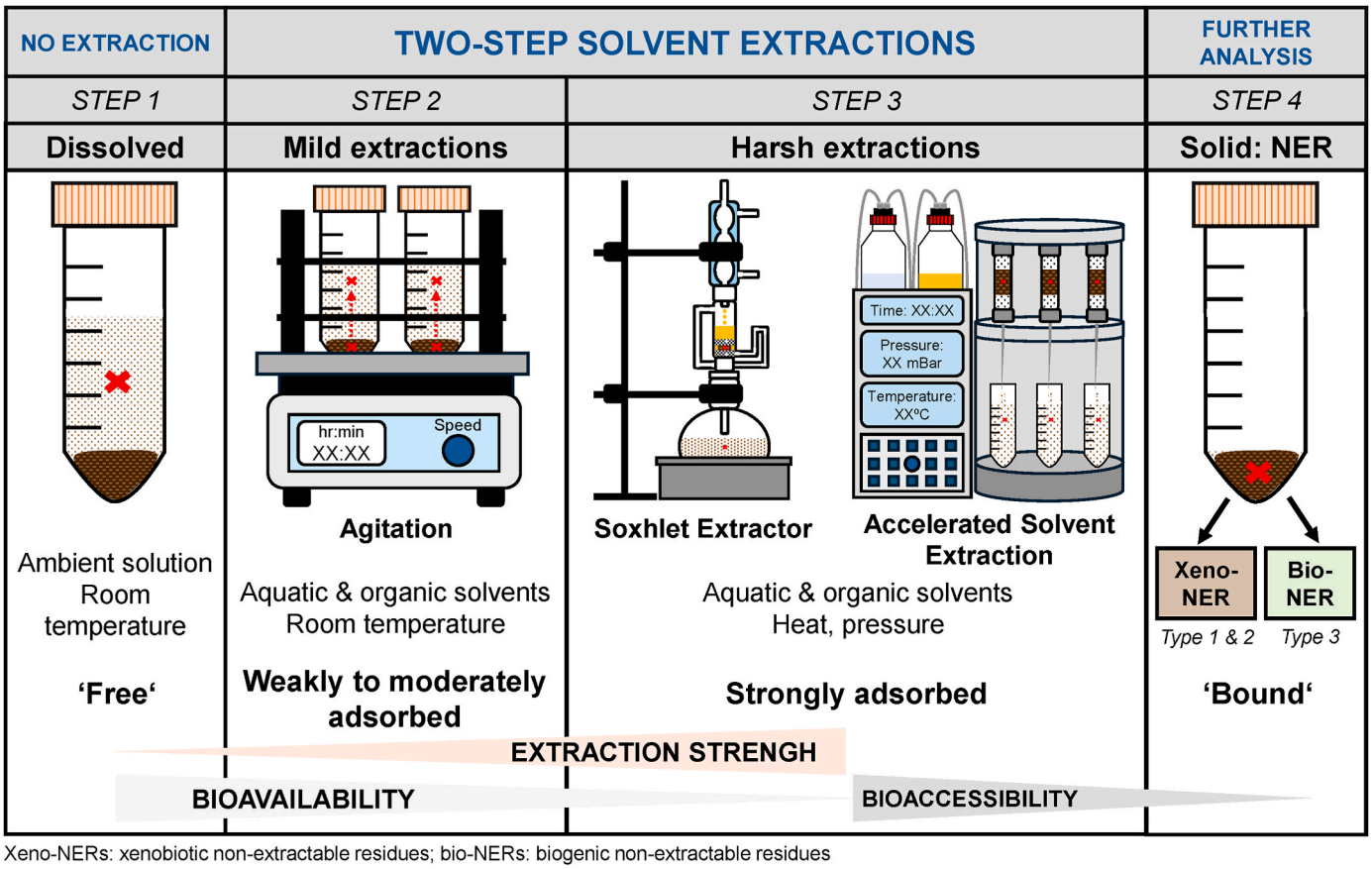


Fig. 5. Three-step approach: no extraction (step 1), mild (step 2) and harsh (step 3) extractions to isolate residues from a sample. The three-step method is also used to estimate bioavailability and bioaccessibility of contaminants to living organisms. After the procedure, the remaining un-extractable isotope label in the solid matrix is attributed to non-extractable residues (NER, step 4), which can be further analysed to differentiate between three types of NER.

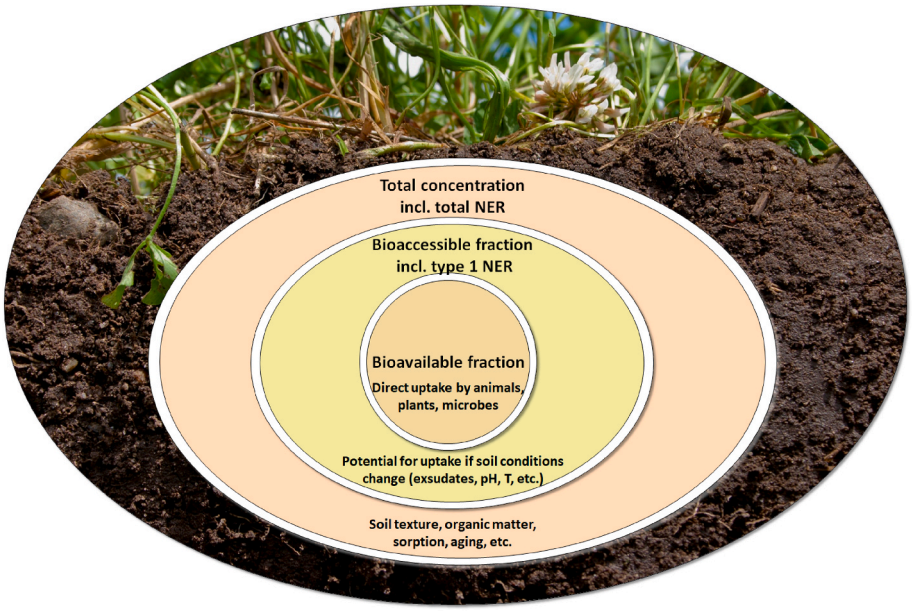


Fig. 6. Total, bioaccessible and bioavailable fractions of contaminants in soil. Methods for determining fractions by extraction procedures are described in Table 1.

biological tissue) [26]. This extraction step can release weakly to moderately adsorbed contaminants onto solids. Extraction methods are summarized in Table 1.

In the last third step, advanced techniques such as Soxhlet extraction, Accelerated Solvent Extraction (ASE), Supercritical Fluid Extraction (SFE), or Microwave-Assisted Extraction (MAE) are employed to release strongly adsorbed residues [26]. These steps are crucial for achieving near-complete removal of extractable fractions. Harsh acidic or alkaline

Table 1
Comparison of chemical and biological methods for assessing bioavailability. Adapted from Semple et al. (2003) [27].

Method	Principle	Strengths	Limitations	Literature
Chemical methods				
Solvent extraction (shown in Fig. 5)				
Step 2: Mild extraction	Aqueous solutions mimicking environmental conditions	Estimates bioavailable fraction; preserves soil integrity	Underestimates hydrophobic compounds	Ortega Calvo et al. (2015) [23]
Step 3: Harsh extraction (e.g., Soxhlet, ASE)	Organic solvents to extract total contaminant mass	High recovery	Overestimates bioavailability	Barriuso et al. (2008) [13]
Other methods				
Passive sampling (often used polymer types: polyethylene, polydimethylsiloxane, polyoxymethylene)	Equilibrium-based partitioning into polymer	Measures freely dissolved concentration; non-destructive	Potentially long equilibration times	Jonker et al. (2020) [28], Mayer et al. (2014) [29]
TENAX extraction	Adsorption	Estimates desorbing (bioavailable) fraction	Labor-intensive; compound-specific	Van Noort et al. (2014) [30], Harmsen et al. (2019) [19]
Cyclodextrin extraction	Hydrophobic contaminant inclusion in cyclodextrin cavities	Rapid	Limited to non-polar compounds	Nelieu et al. (2016) [31]
Biological methods				
Biological uptake, e.g., in earthworms	Contaminant accumulation in living organisms	Direct measure of bioavailability; ecologically relevant	Species-specific; slow uptake	Gevao et al. (2001) [32], Katagi et al. (2015) [33]
Microbial assays (respiration)	Metabolic response (e.g., CO_2 evolution) to contaminants	High-throughput; reflects microbial bioavailability	Sensitive to soil nutrients	Krell et al. (2013) [34]

conditions are avoided to preserve the matrix's organic and inorganic structural integrity. The contaminants released using 'harsh' extraction methods are bioaccessible.

Other chemical approaches, which can be used to assess bioavailability include the use of organic polymers (e.g., TENAX or XAD) or cyclodextrin derivatives (Table 1). However, the suitability of cyclodextrins as bioavailability indicators remains debated due to their ability to form complexes beyond a compound's inherent solubility [35]. Biological methods for assessing bioavailability examine toxicity and uptake mechanisms in organisms resulting in body burdens, for example in earthworms [36]. Microbial assays can be also implemented to check a microbial respiration upon the addition of the test contaminant to a medium [34]. However, all biological tests must align with organismal exposure scenarios to accurately reflect bioavailability.

Chemical methods assume chemical proxies mimicking biological uptake, while biological methods measure direct organismal uptake. Chemical methods excel in speed and standardization but may misrepresent biological accessibility, whereas biological methods provide ecological relevance but are resource-intensive and organism-dependent. The 'harsh' extractions (*step 3*), which estimate the total accessible contaminant load may overestimate environmental risks [23]. 'Mild' extraction (*step 2*), no extraction (dissolved contaminants; *step 1*) and passive sampling methods (Table 1) are generally preferred for the assessment of bioavailability, as they better simulate actual real-scenario conditions [23]. The passive sampling effectively measures 'freely' dissolved concentrations but does not account for residues tightly bound to soil particles that organisms might ingest [37].

Following the exhaustive extraction, the matrix containing only NER (*step 4*) is further analysed to differentiate the three NER types, which are described in detail in next sections.

5. NER chemical identity characterization: a step-wise procedure

The remaining isotope label in the solid matrix after solvent extraction is further subjected either to silylation or EDTA extraction or to amino acid extraction [9] (see Fig. 7). The silylation or EDTA extraction aims at the disruption of soil aggregates releasing the entrapped contaminants inside the tiny soil pores (type 1) [12].

To differentiate type I and type II NER, silylation of the soils or sediment organic matter has been used [18]. Silylation replaces H atoms in functional groups (e.g., –OH, –COOH, $–NH_2$) with trimethylsilyl (–Si

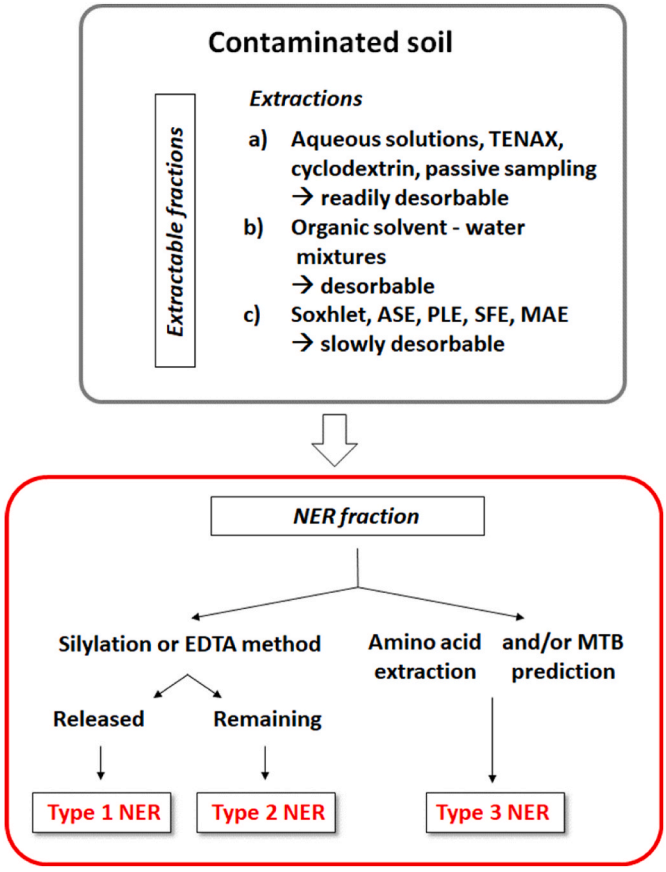


Fig. 7. Sequence of characterization of three NER types (modified from Schäffer et al., 2018[9]). Silylation or EDTA are used to differentiate between the formations of type 1 and type 2 Xeno-NER. The amino acid extraction is used to roughly quantify the total amount of formed type 3 Bio-NER. Fatty acid extraction can be used alternatively for investigating microbial activity and for identifying the major groups of microbial degraders (details see chapter 8). Another way for estimating the amount of Bio-NER is to use the Microbial Turnover to Biomass (MTB) model [21]. The amount of Xeno-NER can be also quantified from the measured total amount of NER minus Bio-NER (measured experimentally or modelled).

$(CH_3)_3$) groups, disrupting H-bridges in metal bridging with humic matter leading to the fractionation of the apparent aggregates into smaller subunits. This process releases physically entrapped residues (type 1 NER), while covalently bound residues (type 2 NER) remain within the matrix (Fig. 8).

Released residues from type 1 NER can be analysed by high performance liquid chromatography-mass spectrometry (HPLC-MS) or gas chromatography-mass spectrometry (GC-MS), depending on the nature of the residues. Type 2 NER are calculated as the total remaining NER minus identified type 1 NER and Bio-NER contributions and can be further analysed for the type of covalent bonds formed between the contaminant and the reactive group of the solid matrix after the bond breakage [9,12]. As an alternative to the silylation procedure, EDTA extractions have been performed to differentiate types 1 and 2 NER [38] (Fig. 7). Another method differentiating between the NER type 1 and 2, is a sequential chemical degradation described in detail in chapter 6.

To quantify the amount of Bio-NER (type 3), i.e., residues incorporated into microbial biomass, the solid matrix is hydrolysed under harsh conditions (e.g., 6 M HCl at 110°C) to release amino acids, formed from the labelled parent substance. The free amino acids are then further quantified with GC-MS and their isotope enrichment is measured with gas chromatography-isotope ratio mass spectrometry (GC-IRMS, for details see chapter 8). The total amino acid content is then used to approximate the total amount of Bio-NER by using a conversion factor of 55% protein content in microbial biomass to enhance reproducibility [3, 12].

Stable isotopes (^{13}C, ^{15}N or D) are best suited for the detailed investigation of Bio-NER formation (Bio-NER type 3) at a biomolecular level. Contrastingly, radioactive isotopes (^{14}C or ^{3}H) are preferred for differentiation between the Xeno-NER type 1 and type 2, since they enable unambiguous and rapid quantitation of radiolabel in liquids or solids. In next sections we discuss in detail applications of both stable and radioactive isotopes for the NER characterization.

6. Sequential chemical degradation to characterize NER types 1 and 2

A strategy to obtain information on the interaction or binding mode of contaminants with the solid matrix, is a sequential application of different chemical degradation techniques. The primary aim of such degradation reactions is to directly disconnect covalently bound NER molecules from the macromolecular natural organic matter. Based on the chemical reaction individual covalent bonds are cleaved with a more or less specificity and strength. The higher the selectivity the higher is the information derived from the resulting reaction products.

First attempts to use sequential chemical degradation have been performed by organic geochemists to obtain information on the molecular structure and linkages of natural macromolecules in both recent and fossil material. As valuable degradation methods, alkaline as well as acidic hydrolysis, ether cleavage by Lewis acids (boron tribromide: BBr_3, hydrogen iodide: HJ) and oxidation reactions with various agents such as RuO_4 or MnO_4 have been mainly applied [39–41]. The selectivity of degradation decreases from moderate conditions such as hydrolysis, disconnecting mainly esters and amides, over more intensive reactions such as ether cleavages towards very harsh treatments with oxidizing reagents attacking carbon-carbon bonds with different dominance for aliphatic or aromatic moieties (Fig. 2).

Noteworthy, not only the directly linked contaminants become deliberated but also the natural macromolecular organic matter becomes altered und partly disconnected by the chemical treatment. Hence, also encapsulated or enclosed proportions of NER become available for common extraction procedures. This is known for natural products [42] but certainly is also valid for anthropogenic contaminants. Consequently, application of degradation reactions allows the analysis of two different pools of NER, the specifically covalently bound compounds (Xeno-NER type 2) as well as incorporated or entrapped ones linked by physical interactions (Xeno-NER type 1). Differentiation of these two fractions can be done by applying a specific cleavage mechanism to break a specific bond formed between the contaminant and organic matter. For example, BBr_3 is used to cleave ether bonds of pollutants at positions where an ether bond is involved in direct covalent binding. Alkaline hydrolysis cleaves ester bonds releasing carboxylic acids, while oxidative degradation techniques let out oxidized species as products. Examples for the bond breakage between DDT and organic matter in sediments using alkaline hydrolysis, BBr_3 treatment and RuO_4 oxidation and the corresponding reaction products are shown in Fig. 9

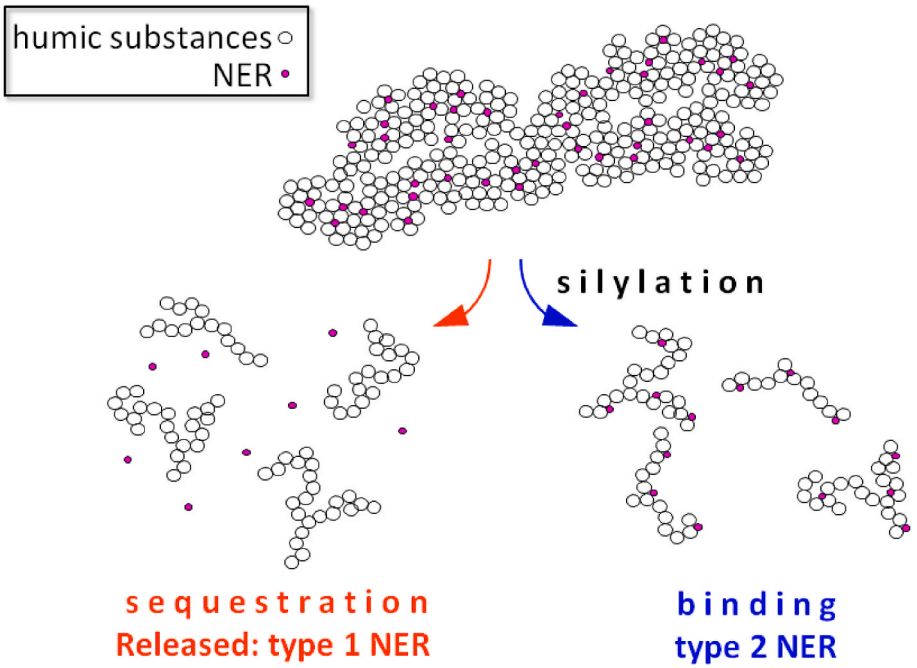


Fig. 8. Humic substances (large white circles) containing non-extractable residues (small pink circles) disintegrate upon silylation. Released residues are considered type 1 NER, those still bound to fractionated humic substances are NER type 2.

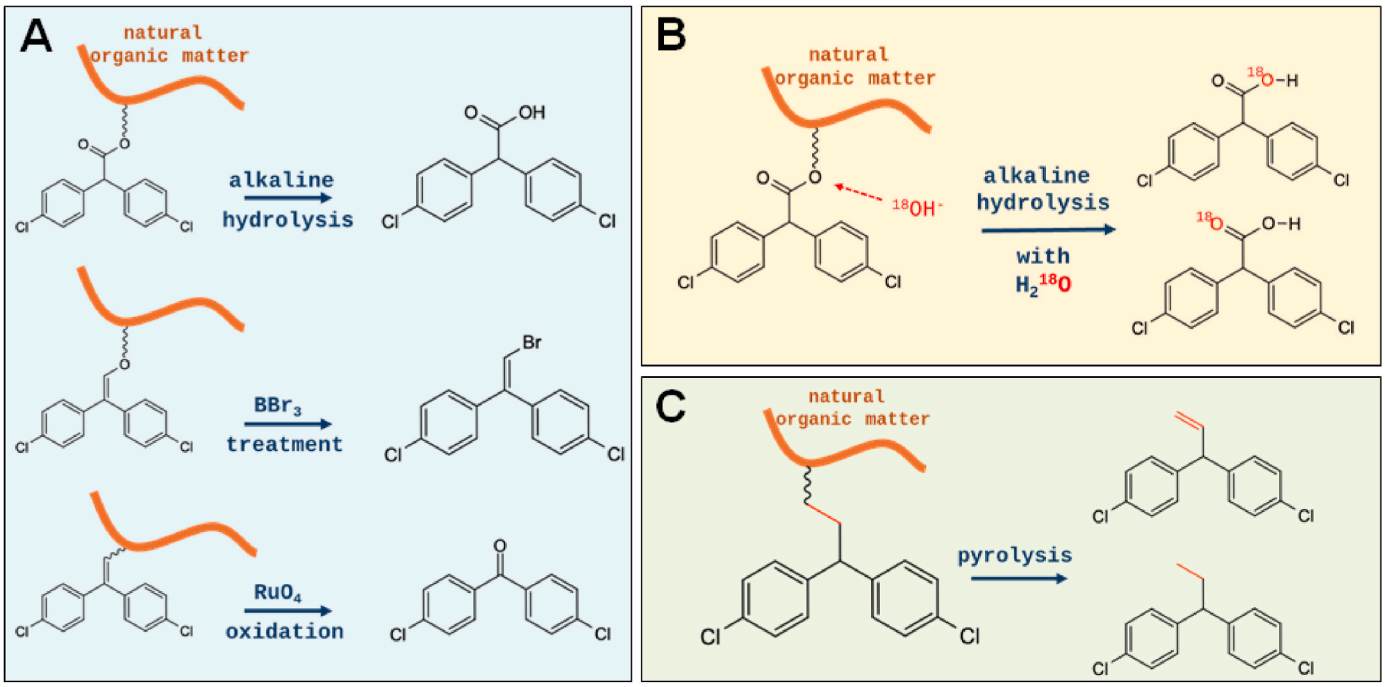


Fig. 9. **(A)** Schematic illustration of cleavage of DDT residues covalently bound to natural organic matter by different chemical degradation methods: alkaline hydrolysis, BBr_3 treatment and RuO_4 oxidation. Adapted and simplified from Zhu et al. (2019) [43]; **(B)** Approach of using labelled chemicals for an unambiguously identification of covalent NER bonds: Cleavage of bound DDA (a metabolite of DDT) by alkaline hydrolysis using ^{18}O-labelled water. Adapted and simplified from Kalathoor et al. (2015) [44]; **(C)** Evidence of carbon-carbon bond linkage (marked in red) of DDT-NER in sediments by pyrolysis based analysis. Adapted and simplified from Kronimus et al., 2006 [45].

(according to Zhu et al., 2019) [43].

However, these approaches do not distinguish between the products released from the covalent bonds cleavage (type 2) and from the entrapped NER fraction (type 1). Therefore, different approaches were used to identify covalent bonds. For example, Richnow et al. (1994) [15,16] applied a smart $Na^{18}OH$-labelled approach (alkaline hydrolysis) to break ester covalent bonds and to identify them. Using labelled chemicals like $H_2^{18}O$ as active reaction component allow to mark products specifically providing clear evidence for a former covalent bond. The mass differences introduced by the labelled components allow an unambiguous identification by mass spectrometry. This approach has been applied also on DDT derivatives as illustrated in Fig. 9.

A further advantage of sequential chemical degradation approaches is the correlation of the different fractions of contaminants released by the individual degradation method applied with the increasing strength. The more destructive and powerful the chemical reaction, that is needed to release the individual fraction, the stronger is the linkage of the bound contaminant to the organic matter. However, a limitation of harsh extractions is that potentially not only bound contaminants are released but also the native soil organic matter matrix is altered. This can create artefacts or obscure the original binding mechanisms. But a clear differentiation based on the individual molecular structures is often possible due to the specific structural properties of many anthropogenic contaminants (e.g. chlorine or bromine substituents, sulfone groups, *tert*-butyl moieties etc.).

The different degradation techniques release two different types of bound contaminants; reversibly and non-reversibly bound. Both binding strength and reversibility have certainly also implications for the bioavailability. Since hydrolysis appears also in nature and represents the inverse reaction of the reversible ester or amide formation, NER releasable by this moderate degradation reaction exhibit a high potential to become bioavailable again in a certain time period. Contrastingly, the NER released only after intensive oxidation processes exhibit a very low potential to become remobilised.

Finally, the chemical degradation approach can also be combined with further analytical methods. As an example, enantioselective analyses combined with chemical degradation processes allowed to identify NER formation as an enantioselective incorporation [46,47].

Noteworthy, besides chemical degradation, pyrolytic methods have been used as degradation tool to obtain information on NER, partly in combination with chemical support such as thermochemolysis or by *in-situ* derivatisation [45]. Since pyrolysis represents a very harsh method for degrading organic material, the information obtainable by this approach is limited. However, in some cases interesting information especially related to the occurrence of contaminants bound strongly by carbon-carbon bonds can be provided. An example of the carbon-carbon bond cleavage between DDT and organic matter during pyrolysis is given in Fig. 9.

7. Radioactive isotopes for NER identification: study-case examples

The environmental fate assessment of organic chemicals often relies on the use of radiolabelled test substances, particularly those tagged with ^{14}C or ^{3}H. Among these, ^{14}C is widely preferred due to its chemical stability (long half-life), low-energy β-emission, and the possibility of incorporating the radiolabel into specific, metabolically relevant positions of the test molecule. Therefore, we here show examples for study-cases using ^{14}C-labelled compounds that allow for quantitative and qualitative insights into degradation, NER formation, and mobilisation.

A typical fate study in soil involves the controlled application of a radiolabelled substance to a soil microcosm, followed by periodic sampling and sequential extraction. The extractable radioactivity can be analysed using chromatographic separation by thin-layer chromatography (TLC) or HPLC coupled to radioactivity detectors, i.e., β-radiation radio-scanner and flow-through radioactivity monitors with scintillation flow cells, respectively. These detectors allow for the resolution and quantification of parent compounds and their metabolites directly from chromatographic effluent, providing metabolite profiles and transformation kinetics.

Liquid scintillation counting (LSC) remains the gold standard for quantitative measurement of total radioactivity. It is employed to assess radioactivity in both liquid extracts and combusted solid matrices (e.g., soil residues, TLC and HPLC fractions, or CO_2 trapped in alkaline solutions). Solid samples after extraction are oxidized in a combustion unit and the resulting $^{14}CO_2$ trapped in a scintillation cocktail for quantification. LSC provides high sensitivity (down to a few disintegrations per minute) and broad linearity, making it ideal for mass balance calculations.

Combining HPLC/TLC-radioanalysis with LSC allows for a comprehensive and robust assessment of the chemicals fate in soil (Fig. 10). Such an integrated analytical approach is essential for identifying persistent transformation products, estimating degradation rates and characterizing NER.

Results from studies employing ^{14}C-labels at different positions in the same molecule can vary substantially, with degradation half-lives and NER quantities depending on the stability and transformation of the labelled moiety. Traditionally, NER have been characterized by measuring the radioactivity associated with soil organic matter fractions - fulvic acids, humic acids, and humin. However, this approach offers limited insight into the chemical nature and mechanisms of residue binding. To address this gap, researchers have combined ^{14}C-radiolabelling with structural elucidation methods such as nuclear magnetic resonance (NMR) spectroscopy - using stable isotopes like ^{13}C or ^{15}N - and mass spectrometry (MS). Few examples for Xeno-NER type 1 and 2 identification using combinatory ^{14}C- and ^{13}C or ^{15}N-labelling are presented below.

In order to improve a low sensitivity of ^{15}N NMR s due to a poor signal-to-noise ratio in N-rich matrices, ^{15}N-labelled simazine was added to ^{15}N-depleted compost. The NMR analysis of ^{15}N-NER derived simazine revealed the presence of both N-dealkylation products and transformation products resulting from triazine ring cleavage. Silylation experiments (description of this derivatisation see chapter 6) indicated strong binding of the residues to the matrix, with only minimal release observed [48].

Sequestration, but not covalent binding of the fungicide dithianon to humic acids was demonstrated using the combinatory approach applying ^{14}C and ^{13}C-labelling. The ^{14}C-dithianon formed 63% of total amount of NER in soil after 64 days, which were mainly incorporated into humic fractions. A parallel degradation study with ^{13}C-labelled dithianon in the soil produced from ^{13}C-depleted straw allowed for the ^{13}C NMR analysis of the humic acid fraction. Since no NMR signals of ^{13}C-dithianon in the humic acids were observed, it was suggested that the ^{13}C-labels of the two cyano groups of the molecule were eliminated metabolically during soil incubation. As a result of this cleavage, the remaining transformation products would still be radio-labelled at the four possible positions of the ^{14}C-atoms remaining in the "naphthoquinone"-part of dithianon [49].

As another example of such combinatory analytics, ^{13}C-cyprodinil was applied on ^{13}C-depleted wheat plants. A control experiment applying same amounts of ^{14}C-cyprodinil showed that main portions of the NER were detected in the cellulose, hemicellulose, and lignin fractions. Neither intact cyprodinil nor its transformation products were detected in the lignin fraction in NMR analysis. The transformation products were thus incorporated in the polymer as natural lignin monomers [50]. NER formation of the same molecule in soil was also investigated by use of ^{14}C-and ^{13}C-labelling. ^{14}C-labelling allowed the quantification of ^{14}C-NER and fractionation in organo-clay complexes. NMR signals indicated the sequestration of the unaltered or slightly altered ^{13}C-cyprodinil in the humin fraction. It also showed that the bond between the two aromatic rings of cyprodinil was cleaved, and that each separated aromatic ring was bound to humic acids [51]. The formation of NER was negligible in sterilized soils, indicating a crucial role of microbial activity in the NER formation. Plant uptake studies further confirmed low bioavailability of cyprodinil in soil, with only 0.2–1.2% of ^{14}C-cyprodinil NER recovered in barley [52].

Incorporation of ^{13}C- and ^{14}C-labelled 4-(3,5-dimethylhept-3-yl) phenol in soil organo–clay complexes revealed an increasing

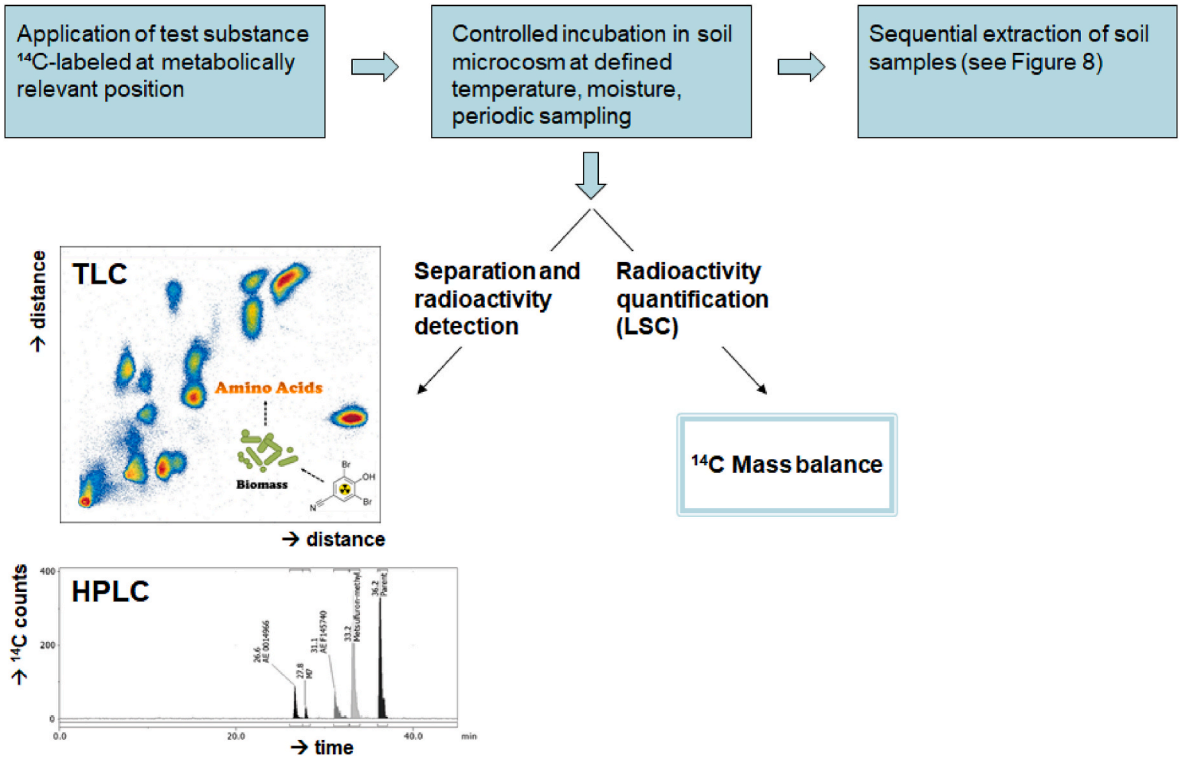


Fig. 10. Schematic representation of a radiolabel-based environmental fate study in soil. A ^{14}C-labelled compound is applied to a soil microcosm and incubated under controlled conditions. Following sequential extraction, the extractable radioactivity is analysed, e.g., via TLC or HPLC coupled with radiodetectors for metabolite profiling. The data enable mass balance calculations and a comprehensive assessment of the chemicals fate.

incorporation of phenolic structures into the NER over time. Further analysis by sequential chemical degradation with the ^{14}C-labelled substance using BBr_3-treatment, RuO_4 oxidation, and thermochemolysis indicated a predominant incorporation of NER via reversible ester (amide) bonds [53].

The antibiotic ^{14}C-sulfadiazine formed rapidly over 90% of NER in soils (of the initially applied ^{14}C). The NER derived from sulfadiazine were found in both fulvic and humic acids, and the latter contained the untransformed ^{13}C-antibiotic as shown by ^{13}C NMR. The antibiotic appeared to be mainly irreversibly bound via covalent binding to soil organic matter. Tight binding of sulfadiazine was also proven in silylation experiments, in which only small amounts of the NER fraction were released [18,54,55]. Difloxacin, another antibiotic, also showed high NER formation (>70% of the initially applied ^{14}C after 56 days) in the clay fraction. ^{14}C-labelling confirmed the presence of the parent compound or transformation products integrated [56] into humic materials which were major components in the NER, across all humic fractions. Laccase-mediated formation of covalent binding was also reported for the first major transformation product of the fungicide metalaxyl, i.e., metalaxyl acid [57].

A study with the ^{14}C-labelled flame retardant tetrabromobisphenol A (TBBPA) in soil showed that the NER formation can be affected by changing redox conditions. Under anoxic conditions, only low mineralisation of ^{14}C-TBBPA was accompanied by the formation of transformation products (bisphenol A: BPA, mono-, di-, and tribrominated BPA) and 35% of NER was observed. After changing to oxic conditions, 42% of the formed NER was released as the parent substance. Thus, sequential anoxic–oxic incubation approaches - often applied in remediation attempts of soils containing halogenated xenobiotics - may not completely remove xenobiotics from environmental matrices [58].

Although ^{14}C-labelling was mainly used for discriminating between the two Xeno-NER types, there was one attempt applying the ^{14}C-labelling to quantify and identify Bio-NER. For that, ^{14}C-bromoxynil was added to a reference soil in order to establish the ^{14}C-mass balance (as shown in Fig. 1) and to track the ^{14}C-Bio-NER formation. To isolate the Bio-NER fraction, soils were Soxhlet-extracted to remove the extractable residues. After the extraction, amino acids in the soils were hydrolysed from soil proteins, then the hydrolysate was purified over cation exchange resin and ultimately analysed by two-dimensional TLC, GC-MS, and LC-MS/MS. This approach enabled the unambiguous identification of several ^{14}C-amino acids. About 71% of the applied ^{14}C remained in the NER, 19% was mineralized to $^{14}CO_2$. Around 14.5% of the NER consisted of amino acid-bound ^{14}C, which corresponded to 6.1% of the initially applied ^{14}C. Considering that microbial proteins constitute roughly 55% of the total microbial biomass-C, the researchers extrapolated that approximately 26% of the total NER after 56 days could be attributed to Bio-NER [7].

8. Application of stable isotopes for Bio-NER (type 3) characterization

8.1. Stable isotopes in mass balance studies

Stable isotope labelling is widely used to track microbial degradation processes of organic substrates at a biomolecular level [59]. In this approach, the atom(s) of the naturally-occurring most abundant stable isotope of a given element in a test substrate are replaced at a specific position (e.g., the one most stable against degradation) by heavier and low-abundant isotope(s) [60]. Different stable isotopes, such as carbon (^{13}C), nitrogen (^{15}N), oxygen (^{17}O or ^{18}O) and deuterium (^{2}H = D) can be used in microbial degradation studies [15,61–63]. However, natural abundances of these isotopes are variable (see Table 2). The ^{13}C has the highest natural abundance of 1.07%, the ^{15}N-natural abundance is 0.37%, while that of ^{17}O is 0.04%, and the ^{18}O about 0.21% [63]. The D-isotope has the lowest abundance in nature (0.01%). Both ^{13}C and ^{15}N are widely used for balancing of chemical fate [61]. Contrastingly, both

Table 2
Examples of elements and their stable isotopes used in the study of microbial degradation of an organic substrate in an environmental sample. Adapted from Nowak et al. (2018) [64]. Schwartz et al. (2016) [65] described the use of ^{18}O labelling in microbial studies but this technique is not covered in detail in our article.

Element	Most abundant isotope	Stable isotope
Hydrogen	^{1}H 99.99%	^{2}H (D) 0.01%
Carbon	^{12}C 98.93%	^{13}C 1.07%
Nitrogen	^{14}N 99.63%	^{15}N 0.37%
Oxygen	^{16}O 99.76%	^{17}O 0.04%
		^{18}O 0.21%

O-isotopes ($^{17/18}$O) are not used in mass balance studies due to their exchangeable and unstable positions in a molecule (e.g., –OH), and both ^{17}O and ^{18}O are used only for tracking active microorganisms in a sample [63]. Therefore, O-isotopes are not further discussed here.

Compared to radioisotopes, stable isotopes are much more abundant in nature [66]. The naturally abundant heavy isotope from the organic matter of a soil or sediment overlaps with the heavy isotope from a substrate. Therefore, this 'overlap' must be subtracted from a sample [64,67,68]. To correct that, unlabelled controls (no substrate and unlabelled substrate) must be incubated in parallel with the labelled substrate (Fig. 11). After addition of the labelled substrate and incubation, isotope mass balance and isotope flow from the substrate to Bio-NER in a given system can be quantified. The stable isotope mass balance is determined in a similar way to radiolabelling approaches. Instead of LSC, an elemental analyser-isotope ratio mass spectrometry (EA-IRMS) is used to quantify the isotope label in mineralisation products (except for $^{13}CO_2$), extractable and non-extractable residues [69].

The EA-IRMS enables both total quantitation of an element and its isotope enrichment. The total CO_2 trapped into 2 M NaOH is quantified using a total inorganic carbon (TIC) analyser while its isotopic enrichment is determined with GC-IRMS [64]. However, prior to the GC-IRMS measurement, entrapped CO_2 in the NaOH-solution must be freed by addition of an acid to a headspace as a gas, whose isotope enrichment is then measured [69]. The total concentration and biomolecule identification of Bio-NER can be determined with GC-MS, whilst the isotopic composition is measured with GC-IRMS. Detailed protocols for Bio-NER analysis are described in section 8.3.

8.2. Bio-NER formation and biomolecules

During growth, microorganisms mine elements like C, H or N from the labelled substrate to build up the chemical compartment of their cell, i.e., biomacromolecules (proteins, nucleic acids, polysaccharides or lipids [2] (Fig. 12). After the death of microorganisms, labelled biomass residues (e.g., proteins, lipids, polysaccharides) are either stabilised in the solid matrix as Bio-NER or recycled by other growing microorganisms. At each consecutive degradation step, labelled mineralisation products (e.g., CO_2, H_2O, N_2O or N_2) are continuously released from the system [61].

The qualitative and quantitative analyses of labelled biomolecules can be used for different purposes [2,64]. Biomacromolecules can reveal phylogenetic or functional information about microorganisms responsible for metabolism of a substrate [60]. The functional or phylogenetic information is contained in the sequence order of monomers in a macro-biomolecule, e.g., amino acids in proteins or nucleotides in DNA or RNA [2]. Small molecules (monomers, see Table 3) like fatty acids can give 'rough' information about the activity of microorganisms and major microbial groups involved in the transformation of the labelled substrate. The most abundant monomers in the microbial cell - amino acids - are used to approximate the total amount of Bio-NER formed from a labelled substrate [3,64,69]. The fatty acids also can be used to quantify the total Bio-NER, but are less suitable than amino acids due to their fast turnover in soils [3]. Here, we discuss in detail only the application of

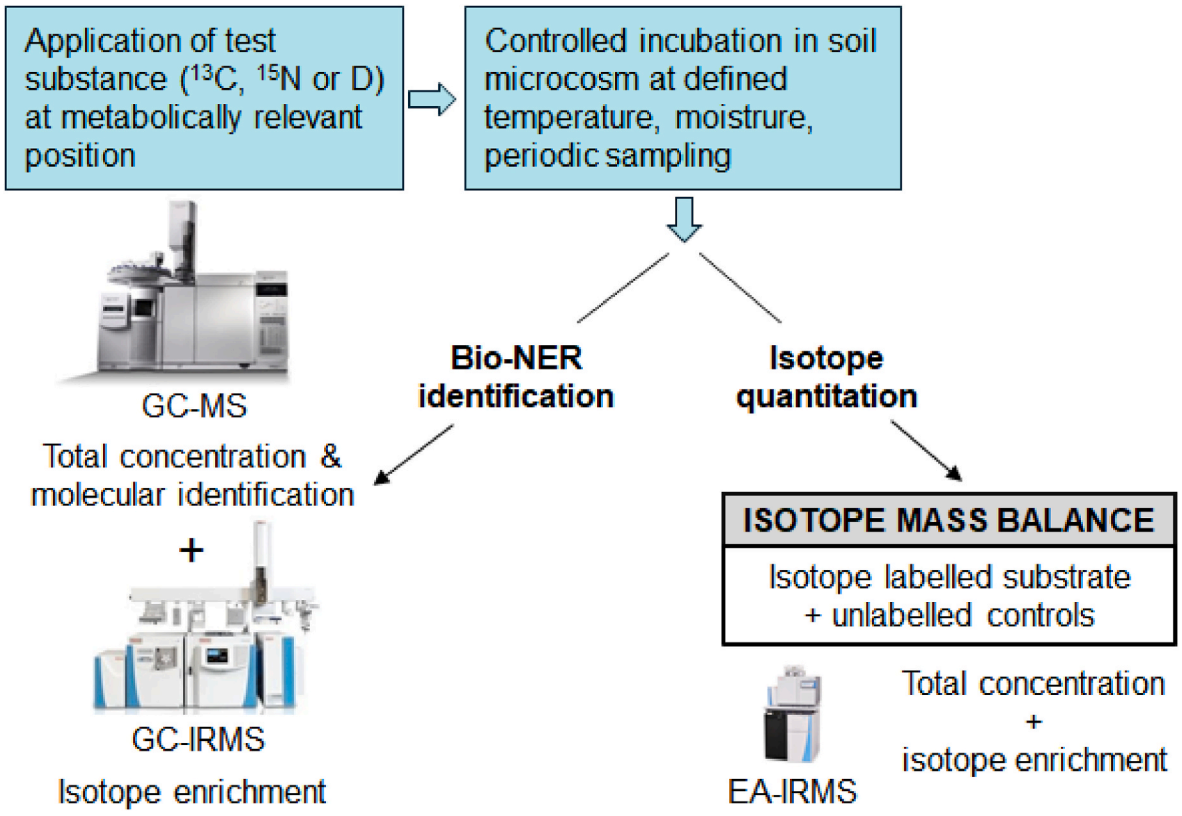


Fig. 11. Schematic representation of a stable isotope-based environmental fate study in soil. A stable isotope labelled compound (e.g., ^{13}C, ^{15}N, or D) is applied to a soil microcosm and incubated under controlled conditions. This set-up enables mass balance calculations and a comprehensive assessment of the chemicals fate. Total elemental concentration and isotope enrichment in a sample is measured with elemental analyser-isotope ratio mass spectrometry (EA-IRMS). Molecular composition of Bio-NER is analysed with gas chromatography-mass spectrometry (GC-MS; total concentration and compound speciation), while the isotope enrichment with gas chromatography-isotope ratio mass spectrometry (GC-IRMS).

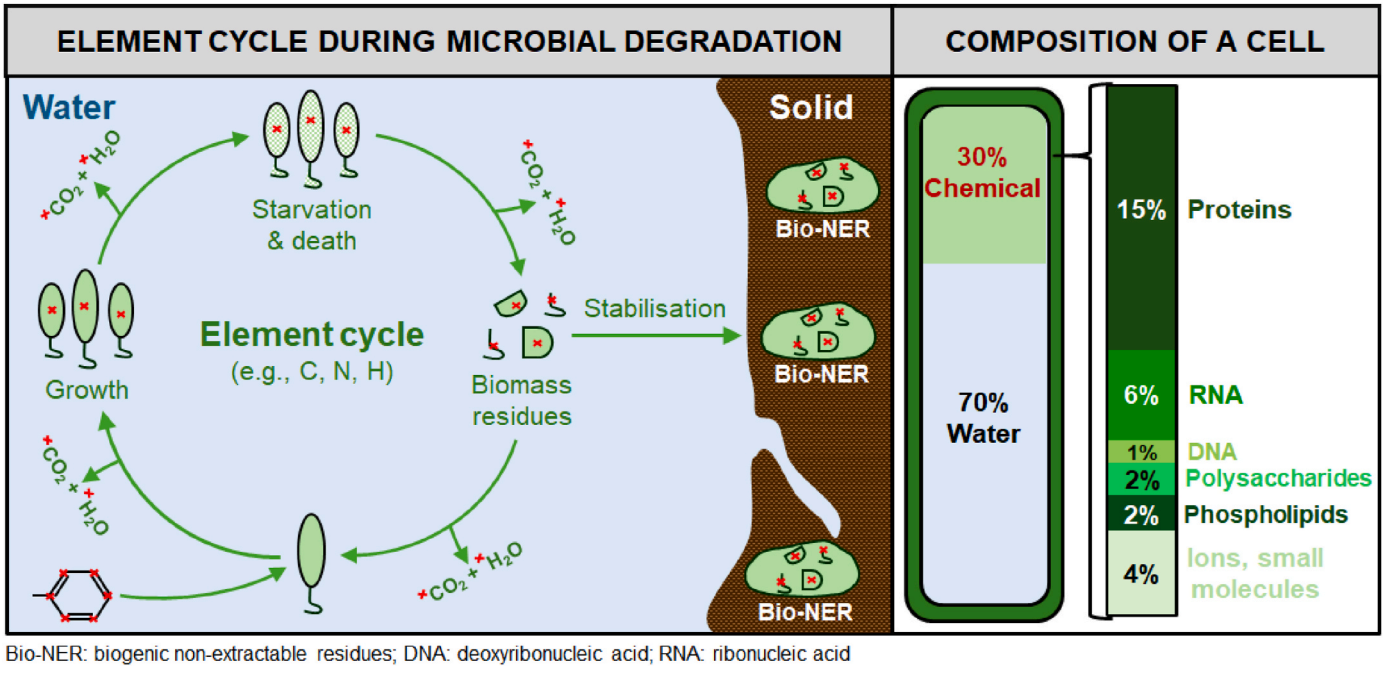


Fig. 12. Element cycle of an isotopically labelled substrate (e.g., ^{13}C, ^{15}N or D) in soil or water-sediment system and the percentage composition of the water and chemical compartment in a microbial cell. During microbial degradation of the isotope labelled substrate, the isotope label can be integrated into microbial biomass, which can ultimately end up in biogenic NER (Bio-NER) or recycled by other microorganisms. Modified from Lennartz et al. (2024) [61].

two labelled monomers - amino acids as a proxy for total Bio-NER, and fatty acids as a proxy for microbial activity, four major groups of

Table 3
The percentage contribution of water and macro-biomolecules in the cell of *Escherichia coli*, *Saccharomyces cerevisiae* and Mammalian cell. Adapted from Delgado et al. (2013) [71].

Component		Monomer	*Escherichia coli*	*Saccharomyces cerevisiae*	Mammalian cell
% of total weight	Water	———	70	80	70
% of dry weight	**Proteins**	Amino acids	50-55	35-60	60
	RNA	Nucleotides	20	6-12	4
	Lipids	Fatty acids	7-9	4-10	13
	DNA	Nucleotides	3	0.1-0.6	1

DNA: deoxyribonucleic acid; RNA: ribonucleic acid.

microbial degraders of a labelled substrate and the total bio-NER. Large macro-biomolecules like proteins or nucleic acids are beyond the scope of this paper; therefore, they are not discussed further.

Amino acids. Amino acids in proteins are the most dominant bio-molecules in the microbial chemical compartment (Fig. 13) and constitute about 50-55% of the total microbial cell dry weight (bacteria) [69,70]. The amino acid content is also comparable in all living organisms (Table 3). Amino acids can be found virtually in every compartment of the microbial cell; cell wall and membrane, pili, flagella, ribosomes, cytoplasm (in particular in enzymes) [70].

Based on the proportions of the amino (-NH_2) and carboxylic (-COOH) groups in the molecule, amino acids can be grouped into three groups. When the amino acid has more –COOH than –NH_2, it is categorised as acidic amino acid like aspartate and glutamate (see Fig. 13). Amino acids with more –NH_2 than –COOH are classified as basic amino acids, e.g., lysine. Amino acids with an equal number of both groups are neutral (major amino acids).

In addition to the highest abundance in the cell, amino acids are also quite stable in the solid matrix. Protein turnover in solids was reported to be relatively slow - half-lives in the range of decades as compared to the bulk C of microbial biomass (half-lives of much less than one year) [72]. Therefore, the analysis of the transfer of isotope labelled C or N in particular into microbial proteins is the most powerful tool to reliably estimate the actual amount of Bio-NER formed in biodegradability testing, especially over long incubation times [2]. However, analysis of amino acids hydrolysed from proteins directly in the soil or sediment sample does not provide any taxonomic information on the degrader community.

Fatty acids. Fatty acids are used for tracing the isotope flux from stable isotope labelled compounds through the microbial degrader communities [73,74]. Phospholipid fatty acids (PLFAs) are one of the constituents in membranes of living cells and storage depots, and make up around 4-10% of the microbial biomass (Table 3) [69,70]. The PLFA pattern of the cell membranes can be used to distinguish between the main groups of microorganisms (Fig. 13). For example, branched PLFAs are characteristic for Gram-positive bacteria or Actinobacteria [75]. One double bond in the PLFAs is indicative for Gram-negative bacteria, whilst two double bonds for fungi [73]. When the PLFA has 'triangle--like' bond, it may suggest the starvation of Gram-negative bacteria [73].

In contrast to amino acids, PLFAs were shown to quickly degrade in

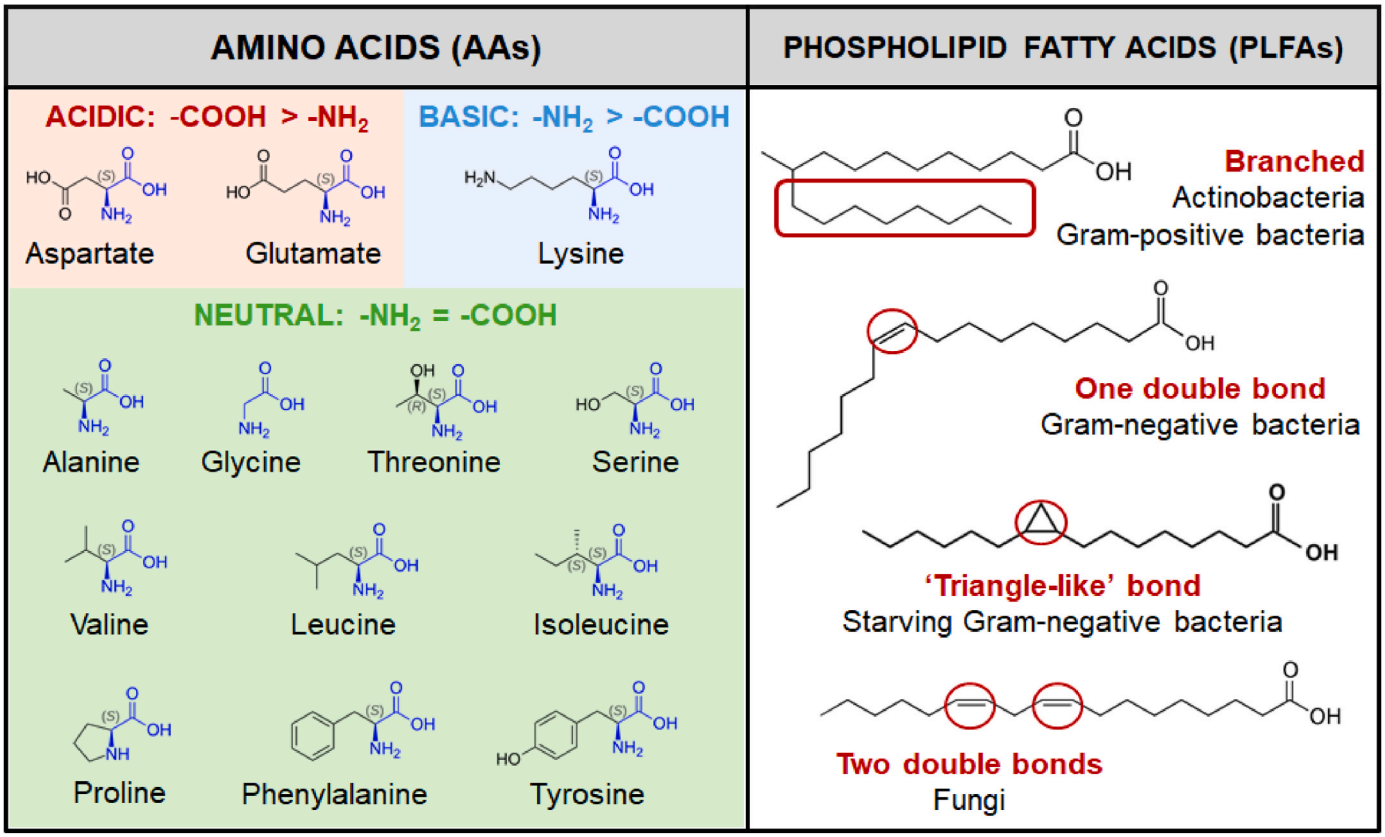


Fig. 13. Classification of amino acids into three groups based on the proportions of –NH_2 and –COOH groups in a molecule (acidic, basic or neutral) and of phospholipid fatty acids based on the structure into four groups (Gram-positive bacteria [including Actinobacteria], Gram-negative bacteria, starving Gram-negative bacteria and fungi).

biodegradability testing with different pesticides [69,76,77]. Due to low abundance and instability in solids, the PLFAs are not very reliable for the quantitation of total Bio-NER, especially over long incubation periods. Instead, the PLFAs analysis is more adequate for the identification of the microbial groups actively involved in the microbial turnover of a substrate and for tracing of the active microbes over time [3]. Due to much lower abundance of PLAFs in soils and sediments compared to amino acids [71], accordingly much lower isotope incorporation into Bio-NER is required to surpass the 'overlap' from natural isotope abundance. Therefore, the PLFAs are more appropriate for quantitation of Bio-NER formed from a slowly biodegradable substrate, from which only low amount of isotope is usually incorporated into the BioNER.

8.3. Extraction protocols and analysis of amino acids and fatty acids

Amino acids (monomers of proteins) and fatty acids (monomers of lipids) are easily analysable biomolecules; they can be measured in two fractions, i.e. total amount in the solid (living + non-living biomass) or in the living biomass of microorganisms [2,8]. This allows estimating the amount of biomass residues (necromass) in organic matter of soils or sediments by difference; i.e., total organic matter minus living biomass [69,76]. The changes in biomolecule contents in the living and non-living fractions over time enable to trace the flux from living biomass to necromass and the decaying microbial residues stabilised in the organic matter [3].

To analyse amino acids in living biomass, the microbial biomass has to be first detached from the solid particles. This can be accomplished by using chelating cation exchange resin and low and high speed centrifugation [78]. Amino acids in solids (or biomass pellets) are hydrolysed from proteins using typically 6 M HCl at 110°C for 22 h [69] (see Fig. 14).

This procedure breaks the peptide bonds of proteins and results in the release of amino acids into the HCl-extract (*step 1*) which must be purified in multiple steps due to high impurity of the hydrolysate (*step 2*) [69]. For that, the hydrolysate is passed through cation exchange resin (DOWEX 50W-X8). Impurities are removed by consecutive washing with 2.5 M oxalic acid, 0.01 M HCl and de-ionized water before eluting samples into 2.5 M NH_4OH solution. Afterwards, the sample is subjected to a two-step derivatisation process (*step 3*): esterification (-COOH groups) and trifluoroacetylation (-NH_2 groups) [79]. After the derivatisation, each amino acid is identified and quantified using GC-MS, which measurement encompasses the quantification of both labelled and unlabelled amino acid fractions (*step 4*). The GC-MS is not sensitive enough to detect trace amounts of a heavy isotope in the sample (<5%). Therefore, more sensitive GC-IRMS is used to detect the trace amounts of a heavy isotope in the range of 0.5 – 2 permille [80]. In the GC-IRMS technique, analyte peaks are separated based on the isotope mass differences (see red colour for a heavy isotope and blue for the light one in Fig. 14), which is recorded as a isotopic ratio (e.g., at% $^{13}C/^{12}C$).

The fatty acids are lysed from the cell membrane with a phosphate buffer/methanol/chloroform mixture (*step 1* in Fig. 14) [81]. Thereafter, the PLFAs are purified from the extract using silica gel using chloroform and acetone and are finally eluted with methanol (*step 2*) [76]. The PLFAs are further subjected to a derivatisation, in which –COOH groups are methylated using methanol + trimethylchlorosilane mixture to fatty acid methyl esters (FAMEs) (*step 3*) [76]. Ultimately, the derivatized PLFAs (FAME) are quantified and identified using GC-MS and their isotope ratio measured with GC-IRMS (*step 4*) [3,69]. In order to analyse total fatty acids (living + non-living fractions) in a soil or sediment, the solid sample first has to be derivatized to FAME prior to the purification step.

8.4. Data analysis: isotope mass balance components and biomolecules

The natural abundance of heavy isotopes (^{13}C, ^{15}N and D), which 'overlap' with a heavy isotope of the introduced substrate, always has to be considered in the isotope mass balance studies with the respective isotope [68]. This abundance is corrected with the help of the controls (I: without substrate and II: unlabelled substrate). The estimation of excess of the respective heavy isotope over the controls in mineralisation products, NER and biomolecules is calculated as shown in equations (1)–(3) for ^{13}C as an example.

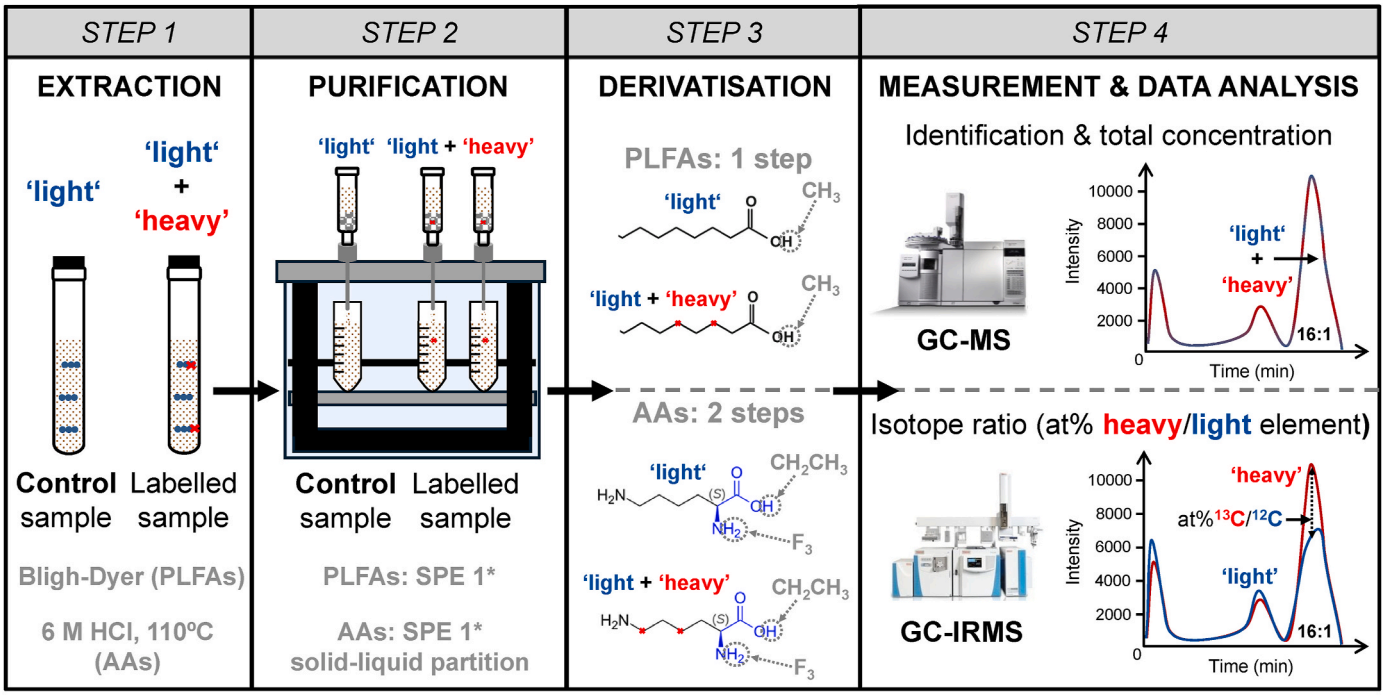


Fig. 14. Four-step protocol of amino acid and fatty acid extraction encompassing purification, derivatisation, measurement and data analysis.

^{13}C control I = C_{OM} × at% $^{13}C_{OM}$ (eq. 1)

^{13}C control II = C_{OM} × at% $^{13}C_{OM}$ + C-unlabelled substrate × at% unlabelled substrate (eq. 2a)

^{13}C labelled = C_{OM} × at% $^{13}C_{OM}$ + ^{13}C-labelled substrate × at% ^{13}C-labelled substrate (eq. 2b)

^{13}C excess = ^{13}C-labelled – ^{13}C control (average of I + II) (eq. 3)

Where.

C_{OM} – total amount of carbon pool in a given fraction originating from the OM
C-unlabelled and C-labelled substrate – total amount of carbon in a given fraction originating from unlabelled and labelled substrate, respectively
at% $^{13}C_{OM}$ – the isotopic signature of OM
at% ^{13}C-unlabelled substrate and at% ^{13}C-labelled substrate – the isotopic signature of unlabelled and labelled substrate, respectively.

During the derivatisation steps (Fig. 14, *step 4*) of amino acids (esterification of –COOH groups) and PLFAs (methylation of –COOH groups) one or more additional carbon/s is/are introduced into the molecule. This shifts the original isotopic composition of the molecule; therefore, these shifts have to be corrected for each PLFA [82] or amino acid [83].

Total Bio-NER contents can be approximated from extracted monomeric biomolecules such as amino acids and fatty acids. Total amino acid hydrolysed from solids are most suitable for approximating the total Bio-NER due to their high stability and abundance in the chemical compartment of microbial biomass (50-55%, Table 3) [69]. In that case, the total amount of amino acid quantified in solids is multiplied by conversion factor of 1.8 (55%) or 2.0 (50%) in order to arrive at approximately 100% amount of Bio-NER.

In certain circumstances (e.g., slow biodegradation of a labelled substrate and thus a low isotope incorporation into Bio-NER), PLFAs can be more suitable for quantifying total Bio-NER. In that case, a conversion factor of 10 is applied (Table 3) [71]. Due to a fast turnover of PLFAs, the conversion factor of 10 might be too low, especially over long incubation periods. Nowak et al. (2011) showed that the factor of 20 might be necessary to arrive at appr. 100% amount of Bio-NER [69].

8.5. ^{13}C, ^{15}N and D in Bio-NER quantitation: comparison

The most common isotope chosen for the stable isotope mass balance of an organic contaminant in biodegradability testing is carbon (^{13}C) [61]. The reason behind this choice is that carbon is universally contained in (bio)organic molecules and C fluxes can be tracked accurately in many biochemical reactions [63], and in Bio-NER. However, the natural abundance of ^{13}C (1.07%, see Table 2) is quite high when compared with that of nitrogen (^{15}N, 0.37%), or deuterium (^{2}H = D, 0.01%) [63]. Both ^{15}N and D are thus more sensitive than ^{13}C for studying microbial degradation since a ^{15}N- or D-labelled substrate can be tested at a lower concentration [61]. However, not every (bio)organic molecule contains N; therefore, the ^{15}N can be used to investigate N metabolism, for example, biosynthesis of N-containing macro-biomolecules such as proteins, RNA, and DNA [63].

The deuterium (D, ^{2}H) isotope has the lowest natural abundance among all stable isotopes, and it is also universally contained in (bio) organic molecules. However, D is the least commonly used isotope in biodegradability testing because it has been shown to exchange with H from water [63]. Additionally, several studies demonstrated that D in the O—D, N—D or S—D bonds is easily exchanged with protons in liquid and gaseous water [84,85] (Fig. 15).

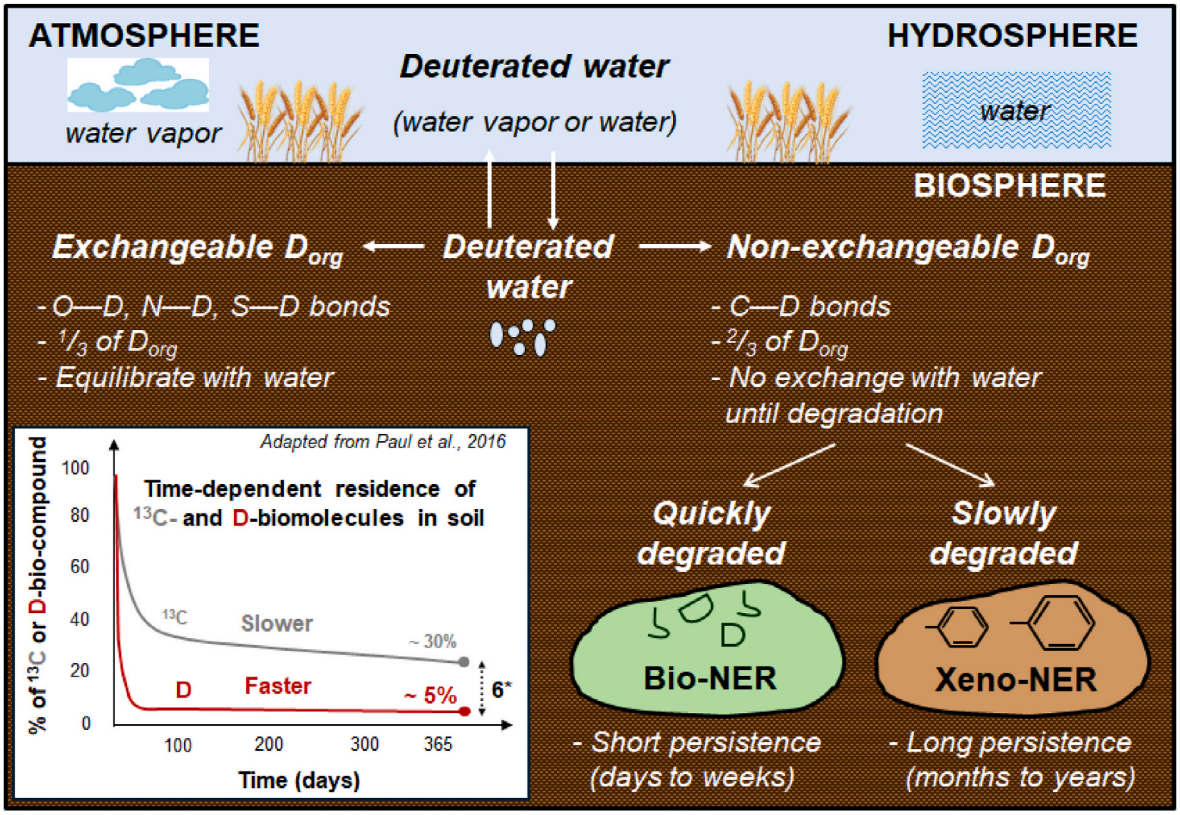


Fig. 15. Total pool of hydrogen (D here) in the environment. Adapted from Eyrolle et al. (2018) [86]. D is exchangeable in the O—D, N—D, S—D bonds, whereas the C—D bonds are mostly stable. Time-dependent residence of D-labelled biomolecules is about six times shorter than that of ^{13}C. Therefore, low formation of Bio-NER is expected when a contaminant is biodegraded. NER: non-extractable residues; bio: biogenic; xeno: xenobiotic.

Contrastingly, no loss of D from the covalent C—D bonds of the two pesticides 2,4-D and glyphosate and the antibiotic sulfamethoxazole was noticed in biodegradability testing in soil [61]. Even the amino acid 2C—D_2-glycine [61,84] and 2C—D_2-alanine [84] resisted acid hydrolysis and derivatisation by esterification. The D atom in the C—D bonds is mainly exchanged with H-water after the enzymatic degradation of a molecule by microorganisms and pH- or metal-dependent catalysis [87]. Recently, Lennartz et al. (2024) [61] showed that the D-isotope is minimally retained in biomolecules forming only low amounts of Bio-NER. Paul et al. (2016)[85 87] also proved that the residence time of D-labelled bio-compounds like glucose, phenylalanine, isoleucine and palmitic acid was about 6 times shorter compared to their ^{13}C-analogues (see the small inserted figure in Fig. 15). Although the innovative D-labelling approach is still not entirely explored yet, it is very promising due to the minimal retention of D in Bio-NER compared to C- and N-isotopes.

During the biodegradation of ^{13}C- or ^{15}N-organic substrates, a big portion of ^{13}C or ^{15}N from not-yet-stabilised biomass residues is constantly recovered by other growing microorganisms, and only slowly released as $^{13}CO_2$ or $^{15}N_2$ [88–90]. Contrastingly, D is released from the C—D bonds to water after the enzymatic cleavage of the D-substrate [61]. Then, the D is diluted with H-water before its re-uptake by other growing microorganisms so that only a minimal amount of the D-isotope will be integrated into new microbial biomass. Of course, the D-incorporation into microbial biomass and consequently into Bio-NER will still occur but at low amounts [91]. After the (minimal) D-integration into microbial biomass, the D-isotope will be quickly lost from solids as D_2O, which is strongly diluted with H-ambient water at each consecutive degradation step (Fig. 14). Thus, the D-loss from microbial biomass residues as D_2O is much faster than for other labelled mineralisation products (e.g., $^{13}CO_2$, $^{15}N_2O$ or $^{15}N_2$).

If the D-substrate is not biodegraded, the D-compound will be immobilised in the soil or sediment matrix as Xeno-NER type 1 or 2 (Fig. 12). Consequently, an application of D-labelling in regulatory biodegradability testing can facilitate the distinction between the formations of Xeno-NER and Bio-NER. The D-labelling approach thus allows to skip the laborious analytics of Bio-NER, which are formed at high amounts from easily biodegradable ^{13}C- or ^{15}N-labelled organic contaminants.

9. Advantages, disadvantages, synergisms of NER characterization methods

The use of isotopically labelled compounds is central to the investigation of NER in regulatory biodegradability testing of organic contaminants. Two major approaches - radiolabelling using radioactive isotopes (e.g., ^{14}C, ^{3}H) and stable isotope labelling (e.g., ^{13}C, ^{15}N, ^{2}H: D) - offer complementary strengths in quantifying and characterizing NER in complex environmental matrices such as soil, sediment, or sludge. Although ^{3}H-labelling approaches were barely used in biodegradability testing, we show here advantages and disadvantages compared to other isotopes, since the ^{3}H-labelling is suggested for future research (chapter 10).

Quantification and Sensitivity: Radiolabelled compounds, particularly those tagged with ^{14}C, offer unparalleled sensitivity. Using LSC as quantification method or accelerator mass spectrometry (AMS), these labels allow researchers to quantify chemicals and their residues at very low levels - down to the fmol/mL to pmol/mL scale [92]. This high sensitivity is particularly advantageous when studying total mass balances and tracing chemicals into the NER. Stable isotope-labelled compounds (e.g., ^{13}C or ^{15}N) also offer robust quantification when coupled with IRMS or HR-MS. However, their sensitivity is generally lower than that of radiolabelled compounds and their quantification is laborious compared to a rapid and easy radioactivity detection with LSC. The quantification of each fraction in stable isotope mass balance (as shown in Fig. 1) requires multiple data analyses (total concentration + isotope enrichment) and calculation steps (equations (1)–(3) in chapter 8.4).

Structural Elucidation and Mechanistic Insights: One major advantage of stable isotopes is their ability to facilitate structural elucidation of transformation products and NER. NMR, HR-MS or IRMS techniques allow for structural characterization and precise tracking of isotope incorporation (e.g., ^{13}C, ^{15}N) into molecular structures. In contrast, radiolabelling techniques provide limited structural information. Radiodetection methods *per se* only indicate the presence of radioactivity, not the chemical identity of the labelled residues. While radiometric detectors can be coupled to chromatographic systems to identify transformation products, these methods lack the specificity of techniques used for stable isotopes. However, radioisotope labelling allows for testing of chemicals at low (environmentally relevant) concentrations compared to stable isotope labelling, i.e., concentrations often too low for IRMS or NMR analyses. A high initial amount of a stable isotope labelled substrate has to be added to a reference soil or sediment for a reliable stable isotope detection in biomolecules against its natural abundance using IRMS or HR-MS. Due to a poor signal-to-noise ratio in C or N-rich matrices in the NMR technique, the reference solid material (soil, sediment or plant) has to be depleted in a heavy isotope to facilitate a good isotopic detection in a sample. An alternative that could combine a sensitive radioisotope detection with the molecular characterisation of NER could be AMS, but this technique is still not developed for analysis of radioisotope ratios (^{12}C/^{14}C) in biomolecules.

Information on the binding mode also of unlabelled contaminants with solid matrices can be obtained with sequential applications of different chemical degradation techniques as described in chapter 6.

NER Characterization and Differentiation: In P-assessment frameworks like those set by the OECD, distinction between different types of NER is essential. Radiolabelling allows direct quantification of total NERs, including covalently bound or sequestered fractions, which are not extractable by conventional solvents. This capability makes radiolabelled compounds the 'gold' standard for regulatory mass balance and persistence studies. Stable isotopes, on the other hand, can provide insights into the molecular composition of NER. For example, ^{15}N-labelled compounds can help track N-incorporation into amino acids or microbial DNA, indicating biogenic transformation. This mechanistic understanding is valuable for risk assessment but may require complementary quantification strategies to fully characterize the mass of NERs.

Environmental Realism and Applicability: A key limitation of radioactive isotopes is their restricted use in outdoor or long-term experiments due to health, safety, and regulatory constraints. Radiolabelled studies must be conducted in strictly controlled laboratory environments with appropriate containment and waste disposal systems. Also specialized field settings on a limited scale are possible. Stable isotopes circumvent these issues. Their lack of radioactivity allows for safe and more flexible applications, including mesocosm, field, or long-term studies. Additionally, stable isotope probing techniques can be used in combination with metagenomic, proteomic, or lipidomic analyses to understand microbial community responses and biotransformation pathways in realistic environmental conditions. Radiolabelling allows studies with environmentally low concentrations while stable isotopes need higher concentrations that might interfere with the microbiome of soils (toxicity).

Health, Safety, and Regulatory Considerations: Radioactive isotopes require stringent safety measures, including personal dosimetry, containment protocols, and special licensing. This adds complexity and cost to experiments. However, their long-standing regulatory acceptance in persistence and degradation studies makes them indispensable in formal submissions for chemical registration. Stable isotopes offer a safe alternative. They can be handled like ordinary chemicals and are free from the regulatory burdens associated with radioactive materials. This makes them particularly attractive for academic or field-based research.

Cost, Availability, and Instrumentation: The availability of ^{14}C-labelled

compounds is generally good for many legacy pollutants, though expensive custom synthesis is needed for newer structures. Costs on average are moderate, especially for high-volume applications. Required instruments (LSC) are specialized but widely available in regulatory and research labs. Stable isotope-labelled compounds can be more expensive, particularly when position-specific labelling is needed. Lead times can be long, and commercial availability is more limited. On the analytical side, stable isotope detection relies on high-end instruments such as IRMS or LC/GC-MS systems, which may not be readily accessible in all labs and require specialized experts able to maintain devices.

Synergistic Use of Both Approaches: Combining radioactive and stable isotopes offers a synergistic path forward. Radiolabels provide comprehensive mass balance data, distinction of NER types, and total NER quantification, while stable isotopes contribute with structural detail and mechanistic insight. Using both can provide a full picture of the environmental fate of pollutants (Table 4, Fig. 16).

10. Research questions

The recent D-labelling application shows great potential for an easy distinction between harmless Bio-NER and potentially hazardous Xeno-NER type 1. Due to a minimal retention of the D-isotope in Bio-NER, the D-labelling allows Bio-NERs analytics to be skipped (see Fig. 3). Therefore, in the D-approach, only silylation or EDTA extraction needs to be done to distinguish between the formations of type 1 and type 2 Xeno-NER. If needed, the amount of Bio-NER can be calculated from total NER minus Xeno-NER or estimated applying the MTB model. The D-isotope released to liquid extract after the silylation or EDTA would be attributed to type 1.

However, D-quantification in the liquid extract can be challenging. The silylation or EDTA reagent contains high amounts of H along with the naturally abundant D-isotope, which will 'mask' the D-isotope originating from D-labelled contaminants. This, in turn will aggravate the D-quantification in liquid extracts. To improve D-quantification, the liquid extract could be evaporated and the remaining D-isotope in a dried sample pyrolyzed to D_2O and quantified using EA-IRMS. However, potential losses of the D from liquid extract during the evaporation cannot be excluded. The solids that still contain the D-isotope (type 2) could be pyrolyzed and quantified easily. The further characterisation of Xeno-NER type 2 is questionable, since methods breaking covalent bonds between the contaminants and solid matrix are very harsh. Due to the harsh nature of these methods, the D-isotope from C–D bonds might be released [87] in addition to D from the target covalent bond, for which the cleavage was designed. In this case, results would be falsified. Due to these constraints, we therefore recommend skipping the detailed characterisation of the NER type 2 and only quantify the D-isotope in the solid.

D-labelling, although promising, is still difficult to transfer into practice, i.e., to be applied by industrial manufacturers. Stable isotope labelling is not a standard method for isotope mass balancing in regulatory biodegradability testing. Therefore, both manufacturers and regulators have no or very limited experience in stable isotope labelling methods. The limited experience in operating and maintenance of the

Table 4
Advantages, disadvantages, and synergies of non-extractable residue (NER) characterization methods using radioactive and stable isotopically labelled chemicals in environmental fate studies.

Feature	Radioactive isotopes		Stable isotopes		
Isotope	^{14}C	^{3}H	^{13}C	^{15}N	^{2}H
Natural abundance (%)	10^{-12}	10^{-18}	1.07	0.37	0.01
Experimental set-up	Easy controls with unlabelled substance		Controls essential for correction of natural abundance of the isotope		
Labelled compounds	Custom synthesis	Rare (custom synthesis)	Custom synthesis	Rare (custom synthesis)	Wide (internal standards)
Safety concern	Radioactive waste (half-life: 5730 years)	Radioactive waste (half-life: 12.3 years)	Not relevant, no regulatory hurdles		
	Health hazard, environmental impact (LSC cocktails), requires licensing and radiological safety protocols				
Instrumentation	LSC (easy handling)		IRMS (expensive, maintenance, specialized experts needed)		
Detection sensitivity	Extremely sensitive (down to sub-nanogram level via LSC or AMS)		High with IRMS or MS, but generally less sensitive than radiolabels		
Mass balance determination	'Gold' standard	Uncommon (biochemical studies)	Optional (Bio-NER characterization)	Rare (N-molecules)	Uncommon (biochemical studies)
Xeno-NER identification (type 1 vs 2)	Easy quantitation of released radiolabel, no structural information; silylation extract: MS identification	Unexplored yet, risk of the C–^{3}H bond breakage (harsh conditions)	^{13}C quantitation in extracts hard (^{13}C release from solids), supports identification of metabolites and NER origin via MS/NMR	Barely tested (N-molecules)	Unexplored yet, risk of the C–^{2}H bond breakage (harsh conditions)
Bio-NER identification	Possible, but laborious (TLC, radio-HLPC)	Unexplored yet (no Bio-NERs, needs testing)	Optimised methodology (time-consuming)	Optimised methodology (time-consuming)	Can be skipped (low incorporation into biomass)
Quantitative analysis	Easy, fast	Can be challenging (weak β-emitter)	Time-consuming: data from GC-MS (identification + quantitation) + IRMS (isotope enrichment) needed		Time-consuming (can be reduced: no Bio-NERs)
Qualitative analysis	Challenging for biomolecules: high radiolabel integration into biomass needed for radio-UV-HPLC, rough identification with TLC possible		Easy, but time-consuming and expensive		
Data analysis	Quick: quantitation of radioactivity using LSC		Time consuming: both data from GC-MS (identification + quantitation) and IRMS (isotope enrichment) needed. Can be reduced for ^{2}H (no Bio-NER analytics)		
Lab workload for mass balance + NER identification	Medium	Unexplored yet, potentially low (no bioNERs)	High (controls, laboratory work, data analysis)		Medium (controls, no Bio-NERs)
Overall costs	Expensive but lower than for stable isotopes (synthesis for new structures and waste disposal), however ^{14}C-labelled standards available for many compounds		High: extensive lab work, instrumentation, synthesis, limited commercial availability		Medium: instrumentation
Field studies	Limited due to safety and licensing restrictions		compatible with outdoor, mesocosm, and long-term studies (^{13}C,^{15}N)		
Environmental concentrations	Studies with environmentally low concentrations possible		Higher concentrations have to be used because of lower sensitivity of detection methods for stable isotopes		
Regulatory acceptance	Widely accepted for persistence and degradation studies (OECD guidelines)		Increasingly recognized for ^{13}C, but still supplementary in regulatory dossiers		

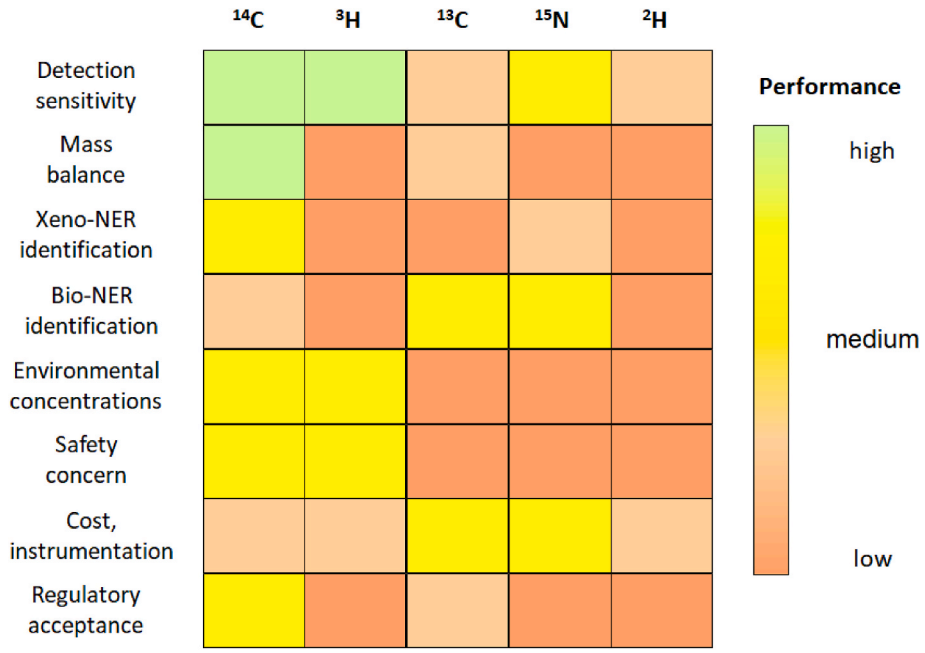


Fig. 16. Applicability of ^{14}C, ^{13}C, ^{15}N, ^{2}H (=D), and ^{3}H (=T) isotopes for NER characterization. Detection sensitivity & Environmental concentrations: radio-isotopes are naturally less abundant than stable isotopes; therefore, lower compound concentration is required. Mass balance: ^{14}C is most applicable, methods for ^{3}H and ^{2}H are not fully optimised yet, quantitation of ^{15}N-mineralisation products is challenging. Xeno-NER identification: isotope quantitation after silylation or covalent bonds cleavage for ^{14}C is easiest, while for ^{13}C challenging due to release of naturally abundant ^{13}C from solid matrix, which overlaps with ^{13}C-substrate. Compared to ^{13}C, ^{15}N is less naturally abundant (thus slightly better performance). The applicability of $^{2/3}H$ for Xeno-NER identification is still unexplored (stability of C-$^{2/3}H$ bonds). Bio-NER identification: ^{13}C and ^{15}N allow detailed molecular characterisation, whilst ^{14}C only allows a 'rough' one. Contrastingly, minimal or no Bio-NER formation is expected for $^{2/3}H$. Safety concern: working with radio-isotopes and waste disposal must follow safety protocols, while stable isotopes are handled as ordinary chemicals. Cost & instrumentation: ^{13}C- and ^{15}N-labelled compounds, analytics and instrumentation are more expensive than for radio-isotopes. No Bio-NER analysis is needed for ^{2}H, which reduces costs and instrumentation (GC-IRMS). Regulatory acceptance: ^{14}C is the gold standard, ^{13}C is also applicable, ^{15}N is limited to N-containing molecules, whilst $^{2/3}H$ is believed to be exchangeable and thus less accepted.

EA-IRMS as well as the complex data analysis are major drawbacks here. The alternative for the D-labelling could be tritium (^{3}H = T)-labelling. T has a very low natural abundance (10^{-16} %) compared to D (0.01%), therefore, quantitative analyses of the T-isotope in the test substance in either liquid extracts or solids will not be constrained by the naturally abundant T-isotope. Another advantage of T-labelling over D-labelling is a reliable and speedy T-radioactivity detection. However, applicability of the T-isotope in mass balancing and in the rapid discrimination between Xeno-NERs and Bio-NERs due to a minimal integration of T in microbial biomass, has not been tested yet. Therefore, further research work could be undertaken in this direction to improve biodegradability testing results using heavy H-isotopes.

Furthermore, it is still little known whether and to which extent the biological or chemical transformation processes of a heavy isotope-labelled contaminant are altered due to mass-dependent isotope effects. The difference of 1 (e.g., ^{13}C, ^{15}N, D) compared to their major isotopes (^{12}C, ^{14}N, ^{1}H) is valid for stable isotopes whilst for radioisotopes difference is 2 (^{14}C versus ^{12}C, ^{1}H versus T). The isotope mass differences between heavy (D or T) and light (^{1}H) isotopes are even more visible for H with a low atomic weight [93]; and this might additionally aggravate the chemical fate compared to C-isotopes with higher atomic weights. It is generally known that light isotopes in the given compound behave slightly different compared to their stable isotopes, e.g., degrade faster than their heavier analogues [80]. This effect, called 'stable isotope fractionation' is widely used to quantify biodegradation of a tested chemical (no isotope labelling, just naturally abundant chemical in heavy isotopes) [94]. This mass-dependent isotope fractionation effect on biodegradation processes is also expected for radioactive isotopes. However, no studies in that direction have been performed so far, but should be done to fill this gap of knowledge.

CRediT authorship contribution statement

Karolina M. Nowak: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Jan Schwarzbauer:** Writing – review & editing, Writing – original draft, Investigation. **Andreas Schäffer:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

Declaration of competing interest

The authors declare to have no conflict of interest.

Acknowledgements

The authors thank the German Research Council (DFG, No 980/3-1; 5471428), the EU project Nowelties (ID 81288) and the Cefic-Long-Range Research Initiative (LRI) which funded the Bio-NER research using stable hydrogen (deuterium) isotope labelling.

Data availability

Data will be made available on request.

References

[1] Z.Y. Wang, G.W. Walker, D.C.G. Muir, K. Nagatani-Yoshida, Toward a global understanding of chemical pollution: a first comprehensive analysis of national and regional chemical inventories, Environ. Sci. Technol. 54 (2020) 2575–2584, https://doi.org/10.1021/acs.est.9b06379.

[2] M. Kaestner, K.M. Nowak, A. Miltner, A. Schaeffer, Multiple) Isotope probing approaches to trace the fate of environmental chemicals and the formation of non-extractable 'bound' residues, Curr. Opin. Biotechnol. 41 (2016) 73–82, https://doi.org/10.1016/j.copbio.2016.05.002.

[3] K.M. Nowak, A. Miltner, M. Kästner, in: J.J. Ortega-Calvo, J.R. Parsons (Eds.), Bioavailability of Organic Chemicals in Soil and Sediment, Springer International Publishing, 2020, pp. 81–111.

[4] ECHA_2017_R.11, Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.11: Endpoint Specific Guidance (PBT/vPvB Assessment), Version 3.0, June 2017.

[5] ECHA-16-B-24-EN. Practical Guide for Smemanagers and REACH Coordinators. How to Fulfil your Information Requirements at Tonnages 1-10 and 10-100 Tonnes per Year, 2016.

[6] OECD, Guidelines for the Testing of Chemicals, Section 3, environmental fate and behaviour. http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-3-degradation-and-accumulation_2074577x.

[7] C. Possberg, et al., Quantitative identification of biogenic nonextractable pesticide residues in soil by C-14-Analysis, Environ. Sci. Technol. 50 (2016) 6415–6422, https://doi.org/10.1021/acs.est.6b00689.

[8] M. Kaestner, K.M. Nowak, A. Miltner, S. Trapp, A. Schaeffer, Classification and modelling of nonextractable residue (NER) Formation of xenobiotics in soil - a synthesis, Crit. Rev. Environ. Sci. Technol. 44 (2014) 2107–2171, https://doi.org/10.1080/10643389.2013.828270.

[9] A. Schaeffer, M. Kaestner, S. Trapp, A unified approach for including non-extractable residues (NER) of chemicals and pesticides in the assessment of persistence, Environ. Sci. Eur. 30 (2018), https://doi.org/10.1186/s12302-018-0181-x.

[10] T.R. Roberts, Non-extractable pesticide residues in soils and plants, Pure Appl. Chem. 56 (1984) 945–956, https://doi.org/10.1351/pac198456070945.

[11] F. Führ, H. Ophoff, P. Burauel, U. Wanner, K. Haider, in: F. Fuhr, H. Ophoff (Eds.), Pesticide Bound Residues in Soil, Wiley VCH, 1998, pp. 175–176.

[12] M. Kästner, S. Trapp, A. Schäffer, Consultancy Services to Support ECHA in Improving the Interpretation of Non-extractable Residues (NER) in Degradation Assessment - a Discussion Paper (Final Report), 1–76, EFSA Report, 2018. https://echa.europa.eu/documents/10162/13630/echa_discussion_paper_en.pdf/4185cf64-8333-fad2-8ddb-85c09a560f7c.

[13] E. Barriuso, P. Benoit, I.G. Dubus, Formation of pesticide nonextractable (bound) residues in soil: Magnitude, controlling factors and reversibility, Environ. Sci. Technol. 42 (2008) 1845–1854, https://doi.org/10.1021/Es7021736.

[14] Z.F. Ren, et al., Microplastics in the soil-groundwater environment: aging, migration, and co-transport of contaminants-A critical review, J. Hazard Mater. 419 (2021), https://doi.org/10.1016/j.jhazmat.2021.126455.

[15] H.H. Richnow, et al., Metabolites of xenobiotica and mineral oil constituents linked to macromolecular organic matter in polluted environments, Org. Geochem. 22 (1994) 671–681, https://doi.org/10.1016/0146-6380(94)90132-5.

[16] H.H. Richnow, et al., Organic pollutants associated with macromolecular soil organic matter: mode of binding, Org. Geochem. 26 (1997) 745–758, https://doi.org/10.1016/S0146-6380(97)00054-5.

[17] ECHA, Options to Address Non-extractable Residues in Regulatory Persistence Assessment. Background Note, 2019.

[18] D. Hennecke, et al., Silylation: a reproducible method for characterization of non-extractable residues (NER) of organic chemicals in the assessment of persistence, Environ. Sci. Adv. 2 (2023) 424–432.

[19] J. Harmsen, D. Hennecke, K. Hund-Rinke, J. Lahr, J. Deneer, Certainties and uncertainties in accessing toxicity of non-extractable residues (NER) in soil, Environ. Sci. Eur. 31 (2019), https://doi.org/10.1186/s12302-019-0281-2.

[20] X.J. Zhu, et al., Substantial halogenated organic chemicals stored in permafrost soils on the Tibetan Plateau, Nat. Geosci. 16 (2023) 989, https://doi.org/10.1038/s41561-023-01293-1.

[21] S. Trapp, A.L. Brock, K. Nowak, M. Kästner, Prediction of the formation of biogenic non-extractable residues during degradation of environmental chemicals from biomass yields, Environ. Sci. Technol. 52 (2018) 663–672.

[22] C. Jespersen, S. Trapp, M. Kästner, Non-extractable residues (NER) in persistence assessment: effect on the degradation half-life of chemicals, Environ. Sci. Eur. 36 (2024), https://doi.org/10.1186/s12302-024-01025-1.

[23] J.-J. Ortega-Calvo, et al., From bioavailability science to regulation of organic chemicals, Environ. Sci. Technol. 49 (2015) 10255–10264, https://doi.org/10.1021/acs.est.5b02412.

[24] K.T. Semple, et al., Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated, Environ. Sci. Technol. 38 (2004) 228a–231a, https://doi.org/10.1021/Es040548w.

[25] V.J.G. Houba, E.J.M. Temminghoff, G.A. Gaikhorst, W. van Vark, Soil analysis procedures using 0.01 M calcium chloride as extraction reagent, Commun. Soil Sci. Plant Anal. 31 (2000) 1299–1396, https://doi.org/10.1080/00103620009370514.

[26] R. Nießner, A. Schäffer, Organic Trace Analysis (*Chapter 6*), vol. 357, De Gruyter, 2017.

[27] K.T. Semple, A.W.J. Morriss, G.I. Paton, Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis, Eur. J. Soil Sci. 54 (2003) 809–818, https://doi.org/10.1046/j.1365-2389.2003.00564.x.

[28] M.T.O. Jonker, et al., Ex situ determination of freely dissolved concentrations of hydrophobic organic chemicals in sediments and soils: basis for interpreting toxicity and assessing bioavailability, risks and remediation necessity, Nat. Protoc. 15 (2020) 1800–1828, https://doi.org/10.1038/s41596-020-0311-y.

[29] P. Mayer, et al., Passive sampling methods for contaminated sediments: scientific rationale supporting use of freely dissolved concentrations, Integrated Environ. Assess. Manag. 10 (2014) 197–209, https://doi.org/10.1002/ieam.1508.

[30] P.C.M. van Noort, A. Poot, A.A. Koelmans, Analysis of organic contaminant desorption kinetic data for sediments and soils: implications for the Tenax extraction time for the determination of bioavailable concentrations, Sci. Total Environ. 490 (2014) 235–238, https://doi.org/10.1016/j.scitotenv.2014.05.015.

[31] S. Nelieu, et al., Evaluation of epoxiconazole bioavailability in soil to the earthworm Aporrectodea icterica, Environ. Sci. Pollut. Control Ser. 23 (2016) 2977–2986, https://doi.org/10.1007/s11356-015-5270-4.

[32] B. Gevao, C. Mordaunt, K.T. Semple, T.G. Piearce, K.C. Jones, Bioavailability of nonextractable (bound) pesticide residues to earthworms, Environ. Sci. Technol. 35 (2001) 501–507, https://doi.org/10.1021/es000144d.

[33] T. Katagi, K. Ose, Toxicity, bioaccumulation and metabolism of pesticides in the earthworm, J. Pestic. Sci. 40 (2015) 69–81, https://doi.org/10.1584/jpestics.D15-003.

[34] T. Krell, et al., Bioavailability of pollutants and chemotaxis, Curr. Opin. Biotechnol. 24 (2013) 451–456, https://doi.org/10.1016/j.copbio.2012.08.011.

[35] B.G. Fouda-Mbanga, et al., Green cyclodextrins-derivatives for sustainable remediation of pesticides and heavy metals: a review, J. Mol. Struct. 1328 (2025), https://doi.org/10.1016/j.molstruc.2025.141326.

[36] Y.C. Qi, W. Chen, Comparison of earthworm bioaccumulation between readily desorbable and desorption-resistant naphthalene: implications for biouptake routes, Environ. Sci. Technol. 44 (2010) 323–328, https://doi.org/10.1021/es902899n.

[37] A. Enell, et al., Combining leaching and passive sampling to measure the mobility and distribution between porewater, DOC, and colloids of native Oxy-PAHs, N-PACs, and PAHs in historically contaminated soil, Environ. Sci. Technol. 50 (2016) 11797–11805, https://doi.org/10.1021/acs.est.6b02774.

[38] D. Loeffler, et al., Determination of non-extractable residues in soils: towards a standardised approach, Environ. Pollut. 259 (2020) 113826, https://doi.org/10.1016/j.envpol.2019.113826.

[39] B. Mycke, W. Michaelis, Molecular fossils from chemical degradation of macromolecular organic-matter, Org. Geochem. 10 (1986) 847–858, https://doi.org/10.1016/s0146-6380(86)80022-5.

[40] P. Schaeffer, C. Reiss, P. Albrecht, Geochemical study of macromolecular organic-matter from sulfur-rich sediments of evaporitic origin (Messinian of Sicily) by chemical degradations, Org. Geochem. 23 (1995) 567–581, https://doi.org/10.1016/0146-6380(95)00045-g.

[41] P. Blokker, et al., Chemical structure of algaenans from the fresh water algae Tetraedron minimum, Scenedesmus communis and Pediastrum boryanum, Org. Geochem. 29 (1998) 1453–1468, https://doi.org/10.1016/s0146-6380(98)00111-9.

[42] L.R. Snowdon, J.K. Volkman, Z.R. Zhang, G.L. Tao, P. Liu, The organic geochemistry of asphaltenes and occluded biomarkers, Org. Geochem. 91 (2016) 3–15, https://doi.org/10.1016/j.orggeochem.2015.11.005.

[43] X.J. Zhu, L. Dsikowitzky, S. Kucher, M. Ricking, J. Schwarzbauer, Formation and fate of point-source nonextractable DDT-Related compounds on their environmental aquatic-terrestrial pathway, Environ. Sci. Technol. 53 (2019) 1305–1314, https://doi.org/10.1021/acs.est.8b06018.

[44] R. Kalathoor, M. Zeiner, B. Schmidt, A. Schaffer, J. Schwarzbauer, First evidence for covalent linkage of acidic metabolites of metalaxyl and DDT as non-extractable pesticide residues in soil and sediment, Environ. Chem. Lett. 13 (2015) 431–437, https://doi.org/10.1007/s10311-015-0514-6.

[45] A. Kronimus, J. Schwarzbauer, M. Ricking, Analysis of non-extractable DDT-related compounds in riverine sediments of the Teltow Canal, Berlin, by pyrolysis and thermochemolysis, Environ. Sci. Technol. 40 (2006) 5882–5890, https://doi.org/10.1021/Es0605568.

[46] R. Kalathoor, J. Botterweck, A. Schaeffer, B. Schmidt, J. Schwarzbauer, Quantitative and enantioselective analyses of non-extractable residues of the fungicide metalaxyl in soil, J. Soils Sediments 15 (2015) 659–670, https://doi.org/10.1007/s11368-014-1027-9.

[47] P. Riefer, J. Schwarzbauer, A. Schäffer, T. Klausmeyer, B. Schmidt, First evidence for a stereoselective incorporation of nonylphenol diastereomers in soil-derived organo-clay complexes, Environ. Chem. Lett. 9 (2011) 293–299.

[48] A. Berns, R. Vinken, M. Bertmer, A. Breitschwerdt, A. Schaffer, Use of ^{15}N-depleted artificial compost in bound residue studies, Chemosphere 59 (2005) 649–658, https://doi.org/10.1016/j.chemosphere.2004.10.027.

[49] U. Wanner, P. Burauel, F. Fuhr, Characterisation of soil-bound residue fractions of the fungicide dithianon by gel permeation chromatography and polyacrylamide gel electrophoresis, Environ. Pollut. 108 (2000) 53–59, https://doi.org/10.1016/s0269-7491(99)00201-8.

[50] T. Ertunc, B. Schmidt, H. Kuhn, M. Bertmer, A. Schaffer, Investigation on the chemical structure of nonextractable residues of the fungicide cyprodinil in spring wheat using C-13-C1-phenyl-cyprodinil on C-13-depleted plants - an alternative approach to investigation nonextractable residues, J. Environ. Sci. Health, Part B 39 (2004) 689–707, https://doi.org/10.1081/lesb-200030786.

[51] J. Dec, K. Haider, A. Schaffer, E. Fernandes, J.M. Bollag, Use of a silylation procedure and C-13-NMR spectroscopy to characterize bound and sequestered residues of cyprodinil in soil, Environ. Sci. Technol. 31 (1997) 2991–2997, https://doi.org/10.1021/es970228v.

[52] J. Dec, et al., Formation of soil-bound residues of cyprodinil and their plant uptake, J. Agric. Food Chem. 45 (1997) 514–520, https://doi.org/10.1021/jf960532s.

[53] P. Riefer, et al., Incorporation mechanisms of a branched nonylphenol isomer in soil-derived organo-clay complexes during a 180-Day experiment, Environ. Sci. Technol. 47 (2013) 7155–7162, https://doi.org/10.1021/es304579s.

[54] T. Junge, et al., Characterization of non-extractable C-14- and C-13-sulfadiazine residues in soil including simultaneous amendment of pig manure, J. Environ. Sci. Health, Part B 46 (2011) 137–149, https://doi.org/10.1080/03601234.2011.535371.

[55] B. Schmidt, et al., Fate in soil of C-14-sulfadiazine residues contained in the manure of young pigs treated with a veterinary antibiotic, J. Environ. Sci. Health, Part B 43 (2008) 8–20, https://doi.org/10.1080/03601230701734824.

[56] T. Junge, N. Classen, A. Schaffer, B. Schmidt, Fate of the veterinary antibiotic C-14-difloxacin in soil including simultaneous amendment of pig manure with the focus on non-extractable residues, J. Environ. Sci. Health, Part B 47 (2012) 858–868, https://doi.org/10.1080/03601234.2012.693868.
[57] J. Botterweck, B. Schmidt, J. Schwarzbauer, R. Kalathoor, A. Schaeffer, Enhanced non-extractable residue formation of C-14-metalaxyl catalyzed by an immobilized laccase, Biol. Fertil. Soils 50 (2014) 1015–1024, https://doi.org/10.1007/s00374-014-0923-x.
[58] F.J. Li, et al., Fate of Tetrabromobisphenol A (TBBPA) and Formation of Ester- and ether-linked bound residues in an oxic sandy soil, Environ. Sci. Technol. 49 (2015) 12758–12765, https://doi.org/10.1021/acs.est.5b01900.
[59] B.E. Morris, R. Henneberger, H. Huber, C. Moissl-Eichinger, Microbial syntrophy: interaction for the common good, FEMS Microbiol. Rev. 37 (2013) 384–406, https://doi.org/10.1111/1574-6976.12019.
[60] C.G. Hoogstraten, J.E. Johnson, Metabolic labeling: taking advantage of bacterial pathways to prepare spectroscopically useful isotope patterns in proteins and nucleic acids, Concepts Magn. Reson. 32A (2008) 34–55, https://doi.org/10.1002/cmr.a.20103.
[61] S. Lennartz, H.A. Byrne, S. Kümmel, M. Krauss, K.M. Nowak, Hydrogen isotope labeling unravels origin of soil-bound organic contaminant residues in biodegradability testing, Nat. Commun. 15 (2024), https://doi.org/10.1038/s41467-024-53478-w.
[62] M. Kästner, K.M. Nowak, A. Miltner, A. Schaffer, Multiple) Isotope probing approaches to trace the fate of environmental chemicals and the formation of non-extractable 'bound' residues, Curr. Opin. Biotechnol. 41 (2016) 73–82, https://doi.org/10.1016/j.copbio.2016.05.002.
[63] S. Klein, E. Heinzle, Isotope labeling experiments in metabolomics and fluxomics, Wiley Interdisciplinary Reviews-Systems Biology and Medicine 4 (2012) 261–272, https://doi.org/10.1002/wsbm.1167.
[64] K.M. Nowak, E. Marco-Urrea, L. Adrian, Markierung im mikrobiellen Stoffwechsel mit stabilen Isotopen, Biospektrum 24 (2018) 491–494.
[65] E. Schwartz, et al., Stable isotope probing with 18O-water to investigate microbial growth and death in environmental samples, Curr. Opin. Biotechnol. 41 (2016) 14–18, https://doi.org/10.1016/j.copbio.2016.03.003.
[66] J. Atzrodt, V. Derdau, W.J. Kerr, M. Reid, Deuterium- and tritium-labelled compounds: applications in the life sciences, Angew. Chem., Int. Ed. 57 (2018) 1758–1784, https://doi.org/10.1002/anie.201704146.
[67] K.M. Nowak, et al., Plant litter enhances degradation of the herbicide MCPA and increases formation of biogenic non-extractable residues in soil, Environ. Int. 142 (2020), https://doi.org/10.1016/j.envint.2020.105867.
[68] S. Wang, et al., (Bio)degradation of glyphosate in water-sediment microcosms - a stable isotope co-labeling approach, Water Res. 99 (2016) 91–100, https://doi.org/10.1016/j.watres.2016.04.041.
[69] K.M. Nowak, A. Miltner, M. Gehre, A. Schaffer, M. Kastner, Formation and fate of bound residues from microbial biomass during 2,4-D degradation in soil, Environ. Sci. Technol. 45 (2011) 999–1006, https://doi.org/10.1021/es103097f.
[70] M. Madigan, W. Sattley, J. Aiyer, D. Stahl, D. Buckley, Brock Biology of Microorganisms, Global Edition, Pearson, 2021.
[71] F.F. Delgado, et al., Intracellular water exchange for measuring the dry mass, water mass and changes in chemical composition of living cells, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0067590.
[72] A. Miltner, P. Bombach, B. Schmidt-Brücken, M. Kästner, SOM genesis: microbial biomass a significant source, Biogeochemistry 111 (2012) 41–55.
[73] A. Kaur, A. Chaudhary, A. Kaur, R. Choudhary, R. Kaushik, Phospholipid fatty acid - a bioindicator of environment monitoring and assessment in soil ecosystem, Curr. Sci. 89 (2005) 1103–1112.
[74] L. Zelles, Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review, Biol. Fertil. Soils 29 (1999) 111–129, https://doi.org/10.1007/s003740050533.
[75] A. Frostegard, E. Baath, The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil, Biol. Fertil. Soils 22 (1996) 59–65, https://doi.org/10.1007/bf00384433.
[76] K.M. Nowak, et al., Contribution of microorganisms to non-extractable residue formation during biodegradation of ibuprofen in soil, Sci. Total Environ. 445 (2013) 377–384, https://doi.org/10.1016/j.scitotenv.2012.12.011.
[77] S. Wang, A. Miltner, A.M. Muskus, K.M. Nowak, Microbial activity and metamitron degrading microbial communities differ between soil and water-sediment systems, J. Hazard Mater. 408 (2021), https://doi.org/10.1016/j.jhazmat.2020.124293.
[78] C.S. Jacobsen, O.F. Rasmussen, Development and application of a new method to extract bacterial-DNA from soil based on separation of bacteria from soil with cation exchange resin, Appl. Environ. Microbiol. 58 (1992) 2458–2462, https://doi.org/10.1128/aem.58.8.2458-2462.1992.
[79] A.M. Muskus, M. Krauss, A. Miltner, U. Hamer, K.M. Nowak, Effect of temperature, pH and total organic carbon variations on microbial turnover of 13CA315N-glyphosate in agricultural soil, Sci. Total Environ. 658 (2019) 697–707, https://doi.org/10.1016/j.scitotenv.2018.12.195.
[80] T.B. Hofstetter, et al., Perspectives of compound-specific isotope analysis of organic contaminants for assessing environmental fate and managing chemical pollution, Nat. Water 2 (2024) 14–30, https://doi.org/10.1038/s44221-023-00176-4.
[81] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[82] J.A. Silfer, M.H. Engel, S.A. Macko, E.J. Jumeau, Stable carbon isotope analysis of amino-acid enantiomers by conventional isotope ratio mass-spectrometry and combined gas-chromatography isotope ratio mass-spectrometry, Anal. Chem. 63 (1991) 370–374, https://doi.org/10.1021/ac00004a014.
[83] T.C.W. Moerdijk-Poortvliet, L.J. Stal, H.T.S. Boschker, LC/IRMS analysis: a powerful technique to trace carbon flow in microphytobenthic communities in intertidal sediments, J. Sea Res. 92 (2014) 19–25, https://doi.org/10.1016/j.seares.2013.10.002.
[84] R. Busch, et al., Measurement of protein turnover rates by heavy water labeling of nonessential amino acids, Biochim. Biophys. Acta Gen. Subj. 1760 (2006) 730–744, https://doi.org/10.1016/j.bbagen.2005.12.023.
[85] A. Paul, et al., Hydrogen dynamics in soil organic matter as determined by 13C and 2H labeling experiments, Biogeosciences 13 (2016) 6587–6598, https://doi.org/10.5194/bg-13-6587-2016.
[86] F. Eyrolle, et al., An updated review on tritium in the environment, J. Environ. Radioact. 181 (2018) 128–137, https://doi.org/10.1016/j.jenvrad.2017.11.001.
[87] P. Grocholska, R. Bachor, Trends in the hydrogen-deuterium exchange at the carbon centers. Preparation of internal standards for quantitative analysis by LC-MS, Molecules 26 (2021), https://doi.org/10.3390/molecules26102989.
[88] E. Blagodatskaya, Y. Kuzyakov, Active microorganisms in soil: critical review of estimation criteria and approaches, Soil Biol. Biochem. 67 (2013) 192–211, https://doi.org/10.1016/j.soilbio.2013.08.024.
[89] M.A. Bradford, A.D. Keiser, C.A. Davies, C.A. Mersmann, M.S. Strickland, Empirical evidence that soil carbon formation from plant inputs is positively related to microbial growth, Biogeochemistry 113 (2013) 271–281, https://doi.org/10.1007/s10533-012-9822-0.
[90] K.M. Buckeridge, et al., Environmental and microbial controls on microbial necromass recycling, an important precursor for soil carbon stabilization, Commun. Earth Environ. 1 (2020), https://doi.org/10.1038/s43247-020-00031-4.
[91] G. Wegener, M.Y. Kellermann, M. Elvert, Tracking activity and function of microorganisms by stable isotope probing of membrane lipids, Curr. Opin. Biotechnol. 41 (2016) 43–52, https://doi.org/10.1016/j.copbio.2016.04.022.
[92] S.D. Gilman, et al., Analytical performance of accelerator mass spectrometry and liquid scintillation counting for detection of 14C-labeled atrazine metabolites in human urine, Anal. Chem. 70 (1998) 3463–3469, https://doi.org/10.1021/ac971383v.
[93] J.A. Krauser, A perspective on tritium versus carbon-14: ensuring optimal label selection in pharmaceutical research and development, J. Label. Compd. Radiopharm. 56 (2013) 441–446, https://doi.org/10.1002/jlcr.3085.
[94] Z. Wang, et al., Revealing the biodegradation mechanism of pesticides: emphasizing the role of isotope fractionation and tracing, J. Agric. Food Chem. (2025), https://doi.org/10.1021/acs.jafc.5c05731.